Characterisation of An Antimicrobial Producer Isolated From the Surface of Seaweed

Submitted by

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ABSTRACT

During a screening of seaweed associated marine micro organisms, for their ability to produce antimicrobial compounds, an organism was isolated, producing antibacterial metabolite(s) and was found to be active against gram positive pathogens, multi-drug resistant pathogenic bacteria, and food borne spoilage fungi. The strain was identified as a gram-negative, short rod, which produced red-pigmented colonies and proved to be both catalase and oxidase positive. Ribotyping, indicated that this strain demonstrated a high sequence identity (99%) to *Serratia plymuthica*. Tests with a type strain of *Serratia plymuthica,* revealed that the strain did not produce a similar spectrum of antimicrobial activity. Preliminary characterisation of the inhibitory molecule(s) produced by this marine isolate, demonstrated that it was heat stable, low molecular weight, with pH resistant compound(s). Moreover, the activity was unaffected by prolonged incubation with enzymes such as *Proteinase K. Transposon*, mutagenesis was utilised, in an attempt to identify the genes involved, in antimicrobial production, to hopefully gain an insight into the nature of the antimicrobial compound. Fourteen mutants, unable to produce antimicrobial compound(s) were isolated, and the preliminary sequence analysis of three of the mutants, highlighted that they had transposon insertions within gene(s) homologous to polyketide synthase. Size exclusion chromatography (Gel filtration) was utilised in an attempt to purify and characterise the antimicrobial compound(s).

DEDICATION

I dedicate this thesis to my parents, my half, my children (Razan, Faisal, Rawan and Fahad), and Professor Khalid Jamal al-Layl.

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DECLARATION

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ABBREVIATIONS

AHLs	N-Acylhomoserine lactones
AEC	Anion Exchange Chromatography
ang	Antimicrobial gene(s)
Ap	Ampicillin
Ap^R	Ampicillin resistant
APS	Ammonium Persulfate Solution
BA	Blood Agar
BHI	Brain Heart Infusion Agar
BLAST	Basic Local Alignment Search Tool
CSM	Concentrated spin medium
Da	Daltons
dH_2O	Distilled water
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
EB	Elution Buffer
EDTA	Ethylene Diamine Tetra Acetic Acid
FPLC	Fast Protein liquid Chromatography
h	Hour
hrs	Hours
HCl	Hydrochloric Acid
Km	Kanamycin
Km^R	Kanamycin resistant
LB	Luria Bertani Agar
l	Litres
MA	Milli Ampere
Ml	Millilitres
MIC	minimal inhibitory concentration
mins	Minutes
MRD	Maximum Recovery Diluent
MRSA	Methicillin-resistant *Staphylococcus aureus*
NA	Nutrient Agar
NB	Nutrient Broth
NaCl	Sodium Chloride

NaOH	Sodium Hydroxide
ng	Nano Gram
NRPs	Nnonribosomal peptides
NRPSs	Nonribosomal peptide synthetases
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
pmol	Picomoles
PM	Potential mutant strains
PKs	Polyketides
PKSs	Polyketide synthases
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Rotation per minute
RT	Room temperature
sec	Second
SEC	Size Exclusion Chromatography
sdH_2O	Sterile distilled water
SDS	Sodium Dodecyle Sulphate
SM	The spent culture medium
SSC	Saline-Sodium Citrate
spp	Species
St	Streptomycin
St^R	Streptomycin resistant
TEA	Tris-acetate-EDTA buffer
$Tetr^R$	Tetracycline resistant
TEMED	N,N,N,N,-Tetramethyle-Ethylenediamine
TJP	Transposon Junction Plasmid
TJPs	Transposon Junction Plasmids
TSA	Tryptone Soya Agar
Tris	2-amino-2hydroxymethylpropan-1,3-diol
TSB	Tryptone Soya Broth
VRE	Vancomycin-resistant *Enterococcus*

INTRODUCTION

1. Introduction

1.1 Antimicrobial Agents

In nature, organisms need to strive in order to survive in their own environment. This kind of bioprocess can be achieved by using competitive mechanisms such as the production of extracellular enzymes, toxins and antimicrobial substrates. An Antimicrobial agents are defied as a mixture of chemical substrates produced by certain species of Eukaryotic and Prokaryotic organisms, and they are able to prevent or inhibit the growth of certain predators and competitors such as protozoa, fungi bacteria (Herbert, 1981; Marwick *et al*., 1999). More precisely, the synthetic chemical or natural agents that can act by preventing or inhibiting the growth of other micro-organisms such as viruses, bacteria, fungi or protozoa are antimicrobial agents (Bryskier, 2005).

1.1.1 Antimicrobial agents from Micro-organisms

There are several groups of micro-organisms such as Gram-negative and Gram-positive bacteria, and fungi have the ability of producing antimicrobial substances with no obvious role in the economy of the organism such as extracellular enzymes (chitinase, proteinase, lipase, casinase, gelatinase and DNase); toxins; CO_2; alcohols (ethanol isopropanol); acids (lactic acid and acetic acid); peroxides (hydrogen peroxide); bacteriocins (nisin and natamycin) and antibiotics (Klaenhammer, 1988; Nosova *et al*., 1996; Frankowski *et al*., 2001; Bryskier, 2005). Historically, Bartolomeo Bizio was the first to report antimicrobial activity in 1823; this was against *Serratia marcescens* and involved moulds. In 1876, John Tyndall observed the same phenomenon, demonstrating the inhibitory activity of *Penicillium* (Bryskier, 2005). One year later, Louis Pasteur and Jules F. Joubert described antimicrobial activity between *Bacillus anthracis* and other bacteria (Bryskier, 2005). Subsequently, in 1886, Louis Gutman and Paul Ehrlich observed antiplasmodia activity (inhibiting the growth of the *Plasmodium* spp that causes malaria) by methylene blue and this was the beginning of studies regarding antimicrobial agents. Following on from this, in 1889, Paul Vuillemin invented the term “antibiotic” to describe the antagonism effect between two micro-organisms (Bryskier, 2005).

1.2 Secondary metabolites

Generally, there is a two–stage process for the production of such secondary metabolites compounds: the growth phase and the production phase. The understanding of these stages is important for any bioreactor producing secondary metabolites, which aims to maximise the biomass in a short time with the best conditions for high production (Marwick *et al*., 1999). However, micro-organisms (often fungi and bacteria) are a good source of secondary metabolite compounds particularly antibiotics, with many, studies having been undertaken to discover natural antimicrobial compounds (secondary metabolites) present in micro-organisms.

As a consequence, more than 120 medicines, and the majority of commercially available antibiotics were obtained from micro-organisms (Ahmed *et al*., 2008). According to Marwick et al. (1999), several factors need to be taken into account, which can affect the production of such useful secondary metabolite compounds from micro-organisms. Some of those factors are discussed below:

1.2.1 Control of secondary metabolite compounds by growth media composition

Microbial growth nutrients, for example carbon, nitrogen, phosphate and trace elements affect microbial growth and the production of secondary metabolite compounds (Marwick *et al*., 1999). For instance, using different quantities and types of sugars can affect the production of the secondary substrates. For example, glucose as a carbon source is often used to harvest maximum biomass growth but it dramatically decreases the production of many secondary metabolites (Martin and Demain, 1980). Several studies have taken this further and demonstrated that the chemical composition of the growth media significantly affects antimicrobial activity (Farmer, 1985; Spížek and Tichý, 1995; Marwick *et al*., 1999).

1.2.2 Control of secondary metabolite compounds by physical factors

Physical parameters such as temperature, pH, oxygen, pressure and salt concentrations can also affect both the micro-organisms and their secondary metabolite production (Marwick *et al*., 1999). For instance, exposure to external factors such as temperature

affects the function of internal pathways. According to Moons et al. (2006), changing the environment of an organism may affect the expression of genes and cell signalling processes involved in the production of antimicrobial secondary metabolites. For example, a newly characterised secondary metabolite compound was induced by changing the dissolved O_2 concentrations (DOC) in liquid growth cultures of *Streptomyces paravulus* (Kaiser *et al*., 1994).

1.3 Antibiotics

According to Bryskier (2005)**,** 'An antibiotic' can be defined as a derivative produced by the metabolism of micro-organisms that possesses antibacterial activity at low concentrations and is not toxic to the host'; this does not apply to all compounds with antibacterial activity, for example, penems and benzyle-pyrimidines, are not considered to be antibiotics as these compounds are detrimental to host cells as well (Bryskier, 2005).

1.3.1 The need for new antibiotics

Currently, the increasing prevalence of antibiotic resistant "super-bugs" this and risks associated with food safety are global issues. For example, according to the Centers for Disease Control and Prevention (http://www.cdc.gov), in the United States of America (USA), about 48 million Americans annually become ill from foodborne disease. Table 1.1 shows the majority of bacterial pathogens associated with the causation of foodborne illnesses.

Table 1.1 Numbers of annual estimated cases of bacterial food borne disease in the USA*

Micro-organism	**No. of annual estimated cases (U.S)**
Salmonella, non-typhoidal	1,027,561
Clostridium perfringens	965,958
Campylobacter spp	845,024
Staphylococcus aureus	241,148

*Cited from http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html (27-12-2012).

1.3.2 Antibiotic resistance

The discovery of antibiotics was a turning point in human history. They have been revolutionary in a number of different respects including the ability to save the lives of organisms including those of humans and animals. However, after the introduction of the first classes of antibiotics (penicillin and sulphonamides), several opportunistic and pathogenic bacteria have developed resistance; or are now becoming more resistant to these commercially available antibiotics (Dessen *et al*., 2001; Alanis, 2005). During the three decades following awareness of antibiotic resistance, it has increasingly been recognised as a problem for hospitalised patients. In the 1930s and 1940s, sulfonamide and penicillin resistance occurred via the pathogenic bacterium *Staphylococcus aureus*. Then, in 1944 after the introduction of the streptomycin antibiotic to treat tuberculosis infections, mutated strains of *Mycobacterium tuberculosis* became resistant to this medicine during patient treatment (Davies and Davies, 2010).

As other antibiotics have been discovered and used to treat patients in clinical practice, a similar course of events has arisen. However, the list of multi-drug resistant bacteria has increased. These included β-lactamase-producing *Haemophilus influenzae* and penicillin-resistant *Neisseria gonorrhoeae* (PPNG), during the 1970s (Jaffe *et al*,. 1981; Jorgensen, 1992); the recovery of the multi-drug resistant (MDR) *Mycobacterium tuberculosis* and *methicillin- resistant Staphylococcus aureus* (MRSA) in the late 1970s and 1980s, (Espinal and Laszlo, 2001; Lowy, 1998); in addition to some gram-negative resistant bacterial strains (common enteric and non-enteric organisms) such as *Escherichia coli*, *Vibrio cholera*, *Shigella* sp., *Salmonella* sp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, some of which were associated with foodborne disease in the 1980s and 1990s (Fey and Safranek, 2000; Waterer and Wunderink, 2001).

More recently, the first record for clinically isolated vancomycin resistant *Staphylococcus aureus* (VRSA) was reported in 2002 from the USA (Tiwari and Sen, 2006). In addition, several studies reported certain cases which were related to the spread of resistant bacteria outside of the hospital environment and responsible for community-acquired infections such as resistance of *Streptococcus spp* to the erythromycin antibiotic (Seppälä *et al*., 1995). *Streptococcus pneumonia* is becoming resistant to penicillin (Nuorti *et al*,. 1998); and vancomycin resistant to *enterococci* (VRE) are now relatively common (DeLisle and

Perl, 2003). The excessive use of antimicrobial agents has also become a global issue. For instance, the numbers of identified β-lactamase enzymes have dramatically increased during the introduction of the β-lactam antibiotics (Davies and Davies, 2010) (See Figure1.1).

According to the world Economic Forum (http://forumblog.org/tag/antibiotics), In the European Union (EU), about 25,000 patients annually die from bacterial infection that associated with antibiotic resistant bacteria.

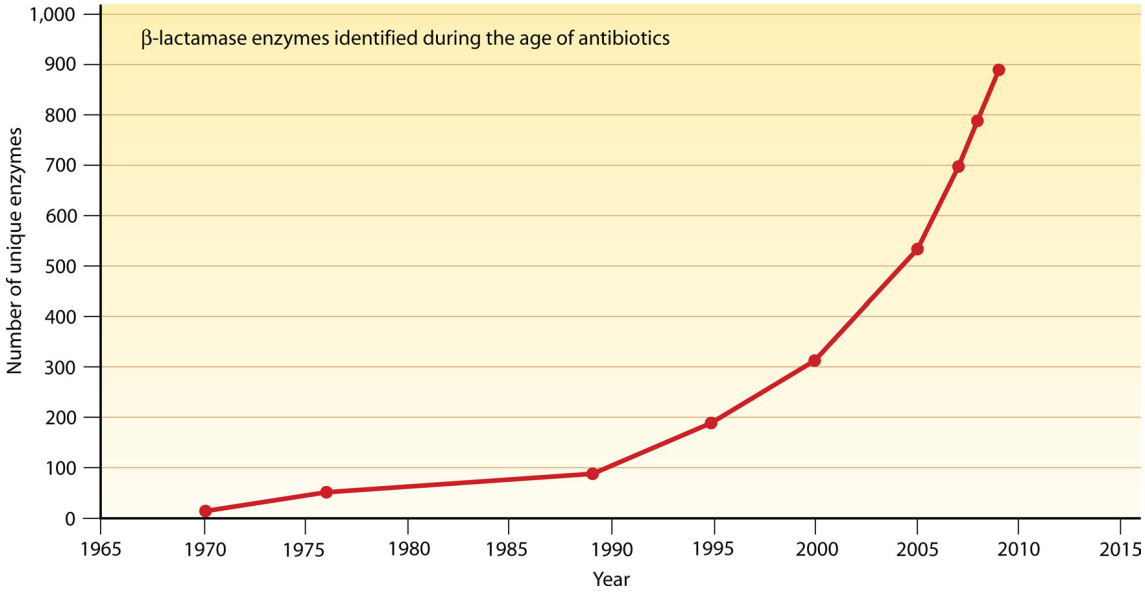


Figure 1.1 The rate of discovery of Beta-lactamase enzymes during the age of antibiotics. From Davies and Davies (2010).

In view of this, new or chemically modified existing antibiotics are needed to prevent the potential spread of multi-drug resistant (MDR) pathogenic bacteria; therefore, several efforts need to be taken into account to reduce antibiotic resistance. Over the years, a number of different solutions have been suggested by international health organisations such as the World Health Organisation (WHO), and the United States Centers for Disease Control and Prevention (CDC). These suggestions have included eliminating the use of antibiotics for the treatment of viral infections and colds; eliminating their use unless there has been an accurate prescription made by a doctor, ensuring that patients take the prescribed dose and complete their course of treatment and also controlled therapeutic use within agriculture and animal husbandry. In addition, there should be an overall awareness regarding disease control through the application of good hygiene by different

countries. These measures should have a substantial impact on reducing the overall spread of disease and eventually the need for antibiotics (Davies and Davies, 2010).

1.3.3 The mechanism of action of antibiotics

Approximately seventeen different classes of antibiotics have been identified for use in clinical practice to date, and at least one mechanism of resistance has developed for each of these groups of antibiotics. The mechanisms of action and modes of resistance of the major antibiotics are summarised in Table 1.2. (Davies and Davies, 2010).

Table 1.2 Mechanisms of action and resistance of commonly used antibiotics (Taken from Davies and Davies, 2010).

Antibiotic families.	**Example.**	**Cellular Process affected.**	**Mode(s) of resistance.**
β-Lactams.	Penicillin (Ampicillin)	Disruption of peptidoglycan biosynthesis (inhibit the transpeptidation by Penicillin-binding Proteins (PBPs)).	β-lactamases and altered target (PBPs).
Aminoglycosides.	Gentamicin and streptomycin	Inhibition of translation (30S ribosomal site).	Mutation and hydrolysis (enzymatic modification).
Glycopeptides.	Vancomycin	Disruption of Peptidoglycan biosynthesis.	Reprogramming peptidoglycan biosynthesis.
Tetracyclines.	Minocycline	Inhibition of translation. (Block tRNA binding to 30S ribosomal site).	Monooxygenation , efflux and altered target.

Table 1.2 continued

Lincosamides.	Clindamycin	Inhibition of translation.	Nucleotidylation, efflux and altered target.
Macrolides.	Erythromycin	Inhibition of translation (50S Ribosome site).	Erythromycin esterase (Hydrolysis), active efflux and altered target.
Streptogramins.	Synercid	Inhibition of Translation.	C-O lyase (Type B streptogramins), acetylation (Type A streptogramins), efflux and altered target.
Oxazolidinones.	Linezolid	Inhibition of translation.	Efflux and altered target.
Phenicols.	Chloramphenicol	Inhibition of ribosomal function (50S ribosomal site) (blocking peptide elongation).	Altered outer membrane (chromosomal mutations).
Quinolones.	Ciprofloxacin.	Inhibit DNA topoisomerases or gyrases (inhibition of DNA replication).	Acetylation, efflux and altered target.
Pyrimidines.	Trimethoprim	Disruption of C1 metabolism.	Efflux and altered target.
Sulfonamides.	Sulfamethoxazole	Disruption of C1 metabolism.	Efflux and altered target.
Rifamycins.	Rifampin	Inhibition of transcription.	ADP-ribosylation, efflux and altered target.

In summary, there is at present a huge demand for new antimicrobial compounds, with lower or no resistance having a wider range of activity and fewer side effects. Two-thirds of the naturally occurring antibiotics discovered so far have been isolated from the terrestrial environment, particularly from cultured soil micro-organisms (Ahmed *et al*., 2008 and Stach, 2010). However, reflecting the increasing importance of seeking out a new source of antimicrobial agents, several studies have been conducted with the aim of investigating new groups of micro-organisms with both industrial and clinical importance form different sources (Wright *et al*., 2001; Xue *et al*., 2003).

One of the key sources of this type of interesting micro-organism is the marine environment; the seas and oceans are known to be highly biodiverse (Bernan and Greenstein, 1997).

1.4 Diversity of natural products from the marine environment

The chances of discovering novel chemical compounds with potential applications for both the industrial and clinical sectors from the marine environment compared to the terrestrial environment are higher, as the water (seas and oceans) cover 70% of the planet (Austin, 1988). The marine environment, often has different organisms to those of found in terrestrial ecosystems (Wright and McCarthy, 1994), and it has a rich biodiversity that presents the necessary growth conditions. These are abundant nutrients, moderate temperatures, light, and pH; in addition, there are a range of conditions such as high salts, low temperatures and high pressure, which may prove ideal for inducing the production of secondary metabolites. Therefore, the reported discovery of new structural classes of compounds from the marine environment is not surprising.

Several studies have indicated that the majority of the antimicrobial compounds, produced from marine micro-organisms, result from secondary metabolites. These metabolites have evolved within certain micro-organisms, in order to help them to survive within the unique conditions of a marine environment (Zheng *et al*., 2005). Furthermore, preliminary studies have suggested that the wealth of microbial diversity within the marine environment makes it an excellent source when searching for new groups of micro-organisms with potential applications for both the industrial and clinical sectors (Xue *et al*., 2003).

1.5 Marine micro-organisms as a source of bioactive compounds

As Jem Stach evoked, "the search for sunken treasure raises images of shiny gold discovered once a seeker's hand wafts away the sand" (Stach, 2010). However, for a different type of treasure hunter, the micro-bioprospector, a marine sample brought from the sea is in itself the goal, whether it comes from the sea-coast, or the depths of the sea, to the laboratory bench. In this case, the micro-bioprospector is interested in marine micro-organisms with biotechnologically important properties; such as industrially relevant enzymes with potential for producing antimicrobial compounds and generating worldwide sales of life-saving products (forecast to be worth around $100 billion USD by 2015) (Stach, 2010). The value of these micro-organisms is inestimably high and this is driving 'prospectors' as if to a gold rush.

Marine micro-organisms form a particular group of organisms which are able to grow in the marine environment, the seas and oceans, and or on, the surface of or inside marine plants or animals, most of which are salt-tolerant with a few being salt requiring. Marine micro-organisms, especially marine bacteria, have been shown to be good producers of bioactive compounds, such as antimicrobial compounds (Davidson, 1995).

The search for novel antimicrobial compounds from marine micro-organisms has been remarkably successful. For instance, in 1947, Rosenfeld and Zobell studied the antibiotic production from marine bacteria; the majority belonged to the *Bacillus* and *Micrococcus* species. Fourteen years later, in 1961 and 1962 further research confirmed the production of antibiotics by marine micro-organisms conducted by Krassil'nikova and Buck et al., respectively (Austin, 1988).

The initial identification and characterisation of antibiotics from marine bacteria took place in 1966 by Burkholder et al, who discovered novel brominated pyrroles from the antimicrobial producing bacterium *Pseudomonas bromoutilis*, which was isolated from *Thalasia* spp. The inhibitory molecule was active against gram-negative pathogenic bacteria; *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. In same year, Lovell confirmed the chemical structure of

this antibiotic as 2, 3, 4-tribromo-5 (1'-hydroxy-2', 4'-dibromophenyl) pyrrole (Lovell, 1966).

In 1974, Anderson et al. isolated an antibiotic producing bacterium from seawater in the North Pacific described as purple pigmented *Chromobacterium* spp., which was named *Chromobacterium marinum* 1-L-33. It produced several inhibitory compounds, extracted with ethyl acetate, and were identified as 4-hydroxybenzaldehyde, hexabromo-2, 2'-bipyrrole, tetrabromopyrrole, and 2-(2'-hydroxy-3' 5'-dibromophenyl)-3, 4, 5-tribromo pyrrole against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* pathogenic yeast *Candida albicans*.

Furthermore, in 1976, Gauthier and Flateau (1976) isolated four antibacterial compounds (two brominated and two polyanionic polysaccharide compounds) from marine violet-pigmented bacterium *Alteromonas luteoviolaceus* with inhibitory activity against *Staphylococcus* human pathogenic bacterium (*Staphylococcus aureus*) and *Staphylococcus epidermidis.*

In 1977, Wratten et al. isolated antibiotic producing yellow-pigmented marine *Pseudomonas* bacterium which produced two novel antibiotics; 2-n-heptyl-4-quinolinol and 2-n-Pentyl-4-quinolinol which inhibited the growth of *Staphylococcus aureus*, *Candida albicans*, *Vibrio anguillarum* and *V. harveyi* (Wratten *et al*,. 1977). Screening for antibiotic producing marine bacteria has continued. In 1985, a study carried out by Lemose *et al*. isolated antimicrobial producer strains from the surface of seaweed. They reported that micro-organisms were found to inhibit the growth of *Staphylococcus aureus* ATCC 25923. They identified the inhibitory molecule found as a low molecular weight inhibitor (smaller than 2KDa). One year later Okami carried out screening for novel antibacterial agents from the marine environment and discovered novel antibiotics. For instance, he discovered new antibacterial agents: Aplasmomycin and Istamycin from marine actinomycetes *Streptomyces griseus* and *Streptomyces tenjimariensis*, respectively, from mud sea samples, which were obtained from the Sagami Bay in Japan (Okami, 1986).

The inhibitory molecule aplasmomycin was active against wide varieties of gram-negative bacteria in addition to *Mycobacterium* spp; whilst the antibiotic Istamycin which belonged to the aminoglycoside family was active against wide varieties of gram-negative

and gram-positive bacteria including micro-organisms that had developed resistance to aminoglycoside antibiotics (Okami, 1986).

A number of investigations of marine bacteria, associated with marine organisms, have also revealed the capability to produce secondary metabolites (the group of metabolites to which the majority of discovered antibiotics, also belong. Shiozawa et al. (1993) isolated antimicrobial antibiotic Thiomarino from the marine isolated bacterial strain *Alteromonas* rava spp. with strong inhibitory activity against gram-negative and gram-positive bacteria. (Shiozawa *et al*., 1993). In 1995, Kunze et al. isolated novel chondramides from marine myxobacteriun *Chondromyces crocatus*, strain Cm c2. The compound showed inhibitory activity against micro-organisms such as yeast in addition to its cytostatic activity against animal tissue cultures including human cells.

A novel antibacterial agent which belongs to the group phenazin antibiotic methyl ester, was isolated from marine actinomycetes *Streptomyces* spp., and termed 5, 10-dihydrophencomycin methyl ester. The compound had antibacterial activity against *E.coli and Bacillus subtilis* (Pusecker *et al*,. 1997). Furthermore, Imamura et al. (1997) discovered a new phenazine antibiotic termed pelagiomicine from a new marine bacterium strain *Pelagiobacter variabilis* Ni-2088, which was isolated from *Pocockiella variegate* (alga). The inhibitory molecule showed good antimicrobial activity against a wide range of gram-positive and gram-negative bacteria including *E. coli*, *B. subtilis*, *P. aeruginosa*, *Enterococcus hirae*, *Salmonella choleraesuis*, *Shigella sonnei*, *Klebsiella pneumoniae* and *S. aureus*.

Gerard et al. (1999) also isolated new decapeptide antibiotics termed loloatins A-D from an unidentified marine bacterium strain (Designated MK-PNG-276A) from the Great Barrier Reef, in Papua, New Guinea. The compound inhibited the growth of multi-drug resistant human pathogenic bacteria, such as vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphyloccoccus aureus* (MRSA) and drug-resistant *Streptococcus pneumoniae*.

In summary, the main conclusion to be drawn from the literature is that the marine environment represents a significant source for isolating micro-organisms, which have the potential to produce novel bioactive metabolites for drug discovery.

Contemporary scientists are engaged in a continual search for antimicrobial compounds with novel characteristics. To date in the region of two-thirds of the bioactive materials present in the environment, that have been taken from bacteria, have been found to have a role to play in medical science; this is especially true of genus actinomycetes (Stach, 2010). The research so far, focusing on identifying marine bacteria, has targeted marine variants of actinomycetes, whilst the investigation of other marine antimicrobial producing bacterial species is relatively limited. However, it is though that by studying isolates from rare marine micro-organisms we will find new producer strains and antimicrobial compounds. Of particular interest to this research are marine variants of the genus *Serratia*, more details of which are given below.

1.6 The genus *Serratia*: taxonomy, distribution and characteristics

Italian physicist Serafino Serrati named the genus *Serratia*; it is a group of bacteria within the Gamma-proteobacteria that relates to both the DNA sequence and phenotypically to the family of Enterobacteriaceae. *Serratia* species are described as gram-negative, rod-shaped, facultative anaerobes and some species (*S. marcescens, S. rubidaea* and *S. plymuthica*) produce a non-diffusible red-pigment (prodigiosin, 2-methyl-3-amyl-6 methoxyprodigiosene). *Serratia* are widely distributed in nature and have been isolated from water (fresh and salt), soil, plants, animals, insects and humans. However, there are several species of the *Serratia*; from those that have a harmful impact on humans such as pathogenic *Serratia marcescens* to those that have a beneficial impact on agriculture in the biological control of pathogenic plant fungi and bacteria as a biocontrol tool such as *Serratia plymuthica*. This genus currently consists of sixteen species and four subspecies, which are listed in Table 1.3 (Grimont and Grimont, 1978; Vleesschauwer and Hofte, 2007; Neupane *et al*., 2012).

Table 1.3: List of *Serratia* species*

Species.	**Type strain.**	**Source/ Reference**
Serratia entomophila	ATCC 43705	Grimont *et al*. (1988)
Serratia ficaria	ATCC 33105	Grimont *et al*. (1981)
Serratia fonticola	ATCC 29844	Gavini *et al*. (1979)
Serratia glossinae	CCUG 57457	Geiger *et al*. (2010)
Serratia grimesii	ATCC 14460	Grimont *et al*. (1982)
Serratia liquefaciens	ATCC 27592	Grimes and Hennerty (1931) and Bascomb *et al*. (1971)
Serratia marcescens	ATCC 13880	Bizio (1823) (Type of genus species)
Serratia marcescens subsp. *ma rcescens*	ATCC 13880	Bizio (1823) and Ajithkumar (2003)
Serratia marcescens subsp. *sak uensis*	CIP 107489	Ajithkumar (2003)
Serratia marinorubra	ATCC 27593	ZoBell and Upham (1944)
Serratia nematodiphila	DSMZ0503SBS1	Zhang *et al*. (2009)
Serratia odorifera	ATCC 33077	Grimont *et al*. (1978)
Serratia plymuthica	ATCC 183	Lehmann and Neumann (1896) and Breed *et al*. (1948)
Serratia proteamaculans	ATCC 19323	Grimont and Grimont (1978)
Serratia proteamaculans subsp *. proteamaculans*	ATCC 19323	Grimont and Grimont (1978) and Grimont *et al*. (1982)
Serratia proteamaculans subsp *. quinovora*	ATCC 33765	Grimont *et al*. (1982)
Serratia quinivorans corrig	ATCC 33765	Grimont *et al*. (1982) and Ashelford *et al*. (2002)

Table 1.3 continued

Serratia rubidaea	ATCC 27593	Stapp, (1940) and Ewing *et al*. (1973)
Serratia symbiotica	DSMZ 23270	Sabri *et al*. (2011)
Serratia ureilytica	CCUG 50595	Bhadra *et al*. (2005)

*Cited from (http://www.bacterio.cict.fr/s/serratia.html) (27-12-2012). Culture Collection, University of Göteborg, Sweden (CCUG), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and the American Type Culture Collection (ATCC).

S. marcescens species is a well known characterised bacterium belong to the genus of *Serratia* spp. Unlike other *Serratia* spp. *S. marcescens* has pathogenic properties. (Grimont and Grimont, 1978). *S. marcescens* is recognised to be an important pathogenic bacterium associated with nosocomial infections and has received intensive attention because of its properties relevant to pathogenicity in humans and its resistance to wide range of antibiotics such as β-lactam antibiotics including ampicillin and aminoglycosides (Sleigh, 1983).

S. marcescens produces a range of metabolite compounds including pigments (red pigment {prodigiosin} and yellow pigment), toxins (lipopolysaccharide{(LPS}), acids (β-cis-cis-carboxymuconic acid and an uncolored muconic acid), enzymes (nuclease, protease, lipase and chitinase) and antibiotics (carbapenam), bacteriocin and siderophore (aerobactin) (Grimont and Grimont, 1978). However, some of these metabolites have antimicrobial properties (antifungal and/or antibacterial activities). These properties have been investigated by several researchers, including Thomson et al. (2000), who investigated the biosynthesis of the antibacterial carbapenam antibiotic (β-lactam antibiotic) that is produced by *S. marcescens* strain ATCC 39006. Also Krausse et al. (2005), reported that *S. marcescens* strain NCTC 10211 has antibacterial activity against the opportunistic human pathogen *Helicobacter pylori* that causes stomach ulcers. This study suggested that *S. marcescens* strain NCTC 10211 has a probiotic activity against *H. pylori*.

1.6.1 Historical aspects of *S. plymuthica*

S. plymuthica is widely distributed and has been isolated from seawater, insects and soil (Grimont and Grimont, 1978), as an opportunistic fish pathogen (Austin and Stobie,

1992) and also from the air of poultry houses (Vucemilo *et al*., 2005). Furthermore, some species of *S. plymuthica* have been found to be associated with nosocomial infections. For instance it has been isolated from surgical wound exudates, blood cultures, the peritoneal fluid, bone marrow infections and central venous catheters (Zbinden and Blass, 1988 and Carrero *et al*., 1995).

According to an internationally approved German directive (TRBA 466), *S. plymuthica* is recognised within risk group 1 by the German Collection of Micro-organisms and Cell Cultures (DSMZ), which indicates that the species does not cause a hazard to human health in comparison to *S. marcescens*, which is grouped in risk group 2. However, so far, there has been insufficient evidence for pathogenicity in *S. plymuthica* when compared to other nosocomial pathogens such as *Stenotrophomonas* and *Burkholderia* (Vleesschauwer and Hofte, 2007). Thus, so far, there is no convincing evidence that *S. plymuthica* is responsible for causing infections to humans.

To date the majority of the work performed on *S. plymuthica* is within the area of soil microbiology and biocontrol and many terrestrial isolates have been extensively studied. Thus it has been isolated from the green part of lettuce and carrot (Grimont and Grimont, 1981) and the rhizosphere of different plants. For instance, the rhizosphere of wheat (Alström and Gerhardson, 1988); tomato (Frommel *et al*., 1991); grape (Chernin *et al*., 1995); oilseed rape (Kalbe *et al*., 1996); maize (Lucon and Melo, 2000); onion (Park and Shen, 2002); Brassica spp. (Carlot *et al*., 2002); Cichorium intybus (Stock *et al*., 2003) and also from the endorhiza of potatoes (Berg *et al*., 2005).

1.6.2 Bioactive compounds produced by the terrestrial isolates of *S. plymuthica*

During the last two decades, several studies have focused on *S. plymuthica* isolates for controlling soil-borne pathogens, particularly fungal plant pathogens. Alström and Gerhardson (1987) isolated the *S. plymuthica* strain G15 from rhizospheres of various plant species. The bacterium showed strong antifungal activity against soil-borne fungal pathogens: *Gerlachia nivalis* and *Botrytis cinerea* and moderate antifungal activity against plant fungal pathogens: *Pythium* spp, *Rhizoctonia solani*. *solani* and *Fusarium culmorum*. One year later they also isolated another strain of *S. plymuthica (*termed A153) from the rhizosphere of wheat (Alström and Gerhardson, 1988). This bacterium was later

used by several researchers in the field of biological control as an antifungal agent producer (Berg, 2000; Levenfors *et al*., 2004).

Chernin et al. (1995) isolated the *S. plymuthica* strain IC1270 from the rhizosphere of grapes. This bacterium inhibited the growth of the soil born fungus *Rhizoctonia solani* on cotton. One year later, the strain of *S. plymuthica* A21-4 was isolated from onion roots and used *in vitro* as biocontrol against the plant fungal pathogen *Phytophthora capsici*. The isolate (*S. plymuthica* A21-4) showed a significant inhibition of cystospore germination, mycelium growth and zoosporangia formation of the pathogenic fungus (McCullagh *et al*., 1996).

Kalbe et al. (1996) carried out a study on 5000 rhizobacteria isolates from the roots of oilseed rape to isolate strains with antifungal properties. The study reported that sixteen strains of the 146 antifungal isolates belonged to *S. plymuthica* strains and showed an antifungal activity against *R. solani*, *Verticillium dahliae* and *Sclerotinia sclerotiorum*. One of the antifungal isolates was well characterised in this study and was termed as the *S. plymuthica* strain HRO-C48. This strain of *S. plymuthica* was also used in another study to evaluate antifungal activity against plant pathogenic fungi *Phytophtora cactorum* and *Verticillium*. The result reported that the *S. plymuthica* strain HRO-C48 caused a significant reduction in fungal disease on strawberry roots, which had resulted from *Verticillium* and *P. cactorum* (Kurze *et al*., 2001).

Galland and Paul (2001) isolated the soil bacterium *S. plymuthica* strain B-781, from the Burgundy region in France. This soil isolate showed strong antifungal activity against the fungal plant pathogen *Pythium perplexum,* which causes damping-off disease of cucumber. More recently, a study performed on the antifungal-producing *S. plymuthica* strain IC1270 showed a good antifungal activity against *Pythium aphaniderma* which causes Pythium disease on cucumbers grown under greenhouse conditions (Ovadis *et al*., 2004).

Berg et al. (2005) isolated an endophyte, *S. plymuthica* (termed B4) from the endorhiza of the potato. This bacterial isolate caused a reduction of up to 25% in the plant disease caused by plant fungal pathogen *R. solani* on potato sprouts. Moreover, the commercial antifungal product (RhizoStar1) has been developed by e-nema GmbH, Raisdorf,

Germany under European patent 98124694.5, from the *S. plymuthica* strain (HRO-C48) (Vleesschauwer and Hofte, 2007).

Only a few studies have reported in reference to using an antifungal producer *S. plymuthica* against post-harvest diseases. For instance, the *S. plymuthica* strain NCIMB40492S=CL43, was used as a biocontrol agent producer (antifungal) against; *Alternaria brassicicola* and *B. cinerea* on Dutch white cabbage in cold storage (Leifert *et al*., 1993). Ritte et al. (2002) reported that the *S. plymuthica* strain IC1270 showed antagonistic activity against the plant fungal pathogens *Penicillium expansum* and *Monilia fructicola*, which cause blue mould on apples and peaches, respectively. Strains of *S. plymuthica* (IC1270 and IC14), have also been used to protect cucumber leaves from fungal diseases such as *Sclerotinia sclerotiorum* (white mould) and *Botrytis cinerea* (grey mould) under greenhouse conditions (Kamensky *et al*., 2003).

This supports a conclusion that over the last two decades strains of *S. plymuthica* from terrestrial sources have been used as biocontrol agents to attack soil-borne pathogens (Fungal pathogens). Although, information regarding *S. plymuthica* from marine isolates is limited. We can also hypothesise in view of current knowledge, that any study of rare marine antimicrobial producer micro-organisms has the potential to lead to the discovery of new antimicrobial compounds. By conducting studies in this area we can increase awareness of the resources in the marine ecosystem, as well as explore the antimicrobial activities of marine *S. plymuthica* strains by testing their effects on foodborne pathogens and multi-drug resistant bacteria, such as VRE and MRSA. More information about the antimicrobial mechanisms *S. plymuthica* is given below.

1.6.3 Antimicrobial mechanisms of *S. plymuthica*

S. plymuthica strains produce a range of antimicrobial metabolites including antibiotics, siderophores and Parasitism (lytic enzymes) (Shoji *et al*., 1989; Chernin *et al*., 1995; Kalbe *et al*., 1996; Faltin *et al*,. 2004; Levenfors *et al*., 2004 and Ovadis *et al*., 2004). An overview of bioactive metabolites produced by strains of *S. plymuthica* is presented in Table 1.4 (Vleesschauwer and Hofte, 2007).

Table 1.4: Overview of antimicrobial metabolites produced by *S. plymuthica* strains (Taken from Vleesschauwer and Hofte, 2007)

***S. plymuthica* strain**	**Metabolite compounds**			
HRO-C48	Pyrrolnitrin	Chitinases	Proteases	Siderophores
IC1270	Pyrrolnitrin	Chitinases	Proteases	Siderophores
IC14	Pyrrolnitrin	Chitinases	Proteases	Siderophores
A153	Pyrrolnitrin	Haterumalides		
R12	Glucanases	Chitinases	Proteases	
3Re4-18	Glucanases	Chitinases	Proteases	Siderophores

1.6.3.1 Antibiotics

Terrestrial *S. plymuthica* isolates have been shown to produce several useful secondary metabolites like antifungal antibiotics. For instance, it is reported that *S. plymuthica* strains may produce antibiotics similar in structure to biosynthesis substrates from type I polyketide synthase, which have a wide range of pharmaceutical potential as anti-tumour intervention and antibiotics (Levenfors *et al*., 2004). However, several studies have reported that terrestrial *S. plymuthica* isolates produce antimicrobial compounds such as chlorinated macrolides, haterumalide (A, B, NE, and X) and pyrrolnitrin, 1-acetyl-7-chloro-1-H-indole and the dipeptide antibiotic CB-25-I.

Shoji et al. (1989), isolated a new antifungal antibiotic, from *S. plymuthica* CB-25, which was isolated from soil samples collected from Fukiagecho and Kagoshima Prefecture, in Japan. The bioactive compound inhibited the growth of *Candida albicans* and was named as CB-25-I and found to be a water-soluble dipeptide. The chemical structure of the compound was identical to the Sch 37137 and A19009 (antifungal antibiotic produced by Actinomycetes strains) (Shoji *et al*., 1989).

Chernin et al. (1996) isolated antibiotics from the *S. plymuthica* IC1270 strain identified as pyrrolnitrin [3-chloro-4-(28-nitro-38-chlorophenyl) pyrrole]. The compound showed inhibitory activity against several phytopathogenic fungi and bacteria. The haterumalides

and chlorinated macrolides were the first polyketide compounds found to be produced by *Serratia plymuthica* strains (Vleesschauwer and Hofte, 2007).

Thaning et al. (2001), isolated from soil a strain of *S. plymuthica* which produced a bioactive compound, which inhibited both ascospore germination and apothecial formation of the fungal plant pathogen *Sclerotinia sclerotiorum*. The compound was identical in chemical structure to chlorinated macrolides and was identified as haterumalide NA.

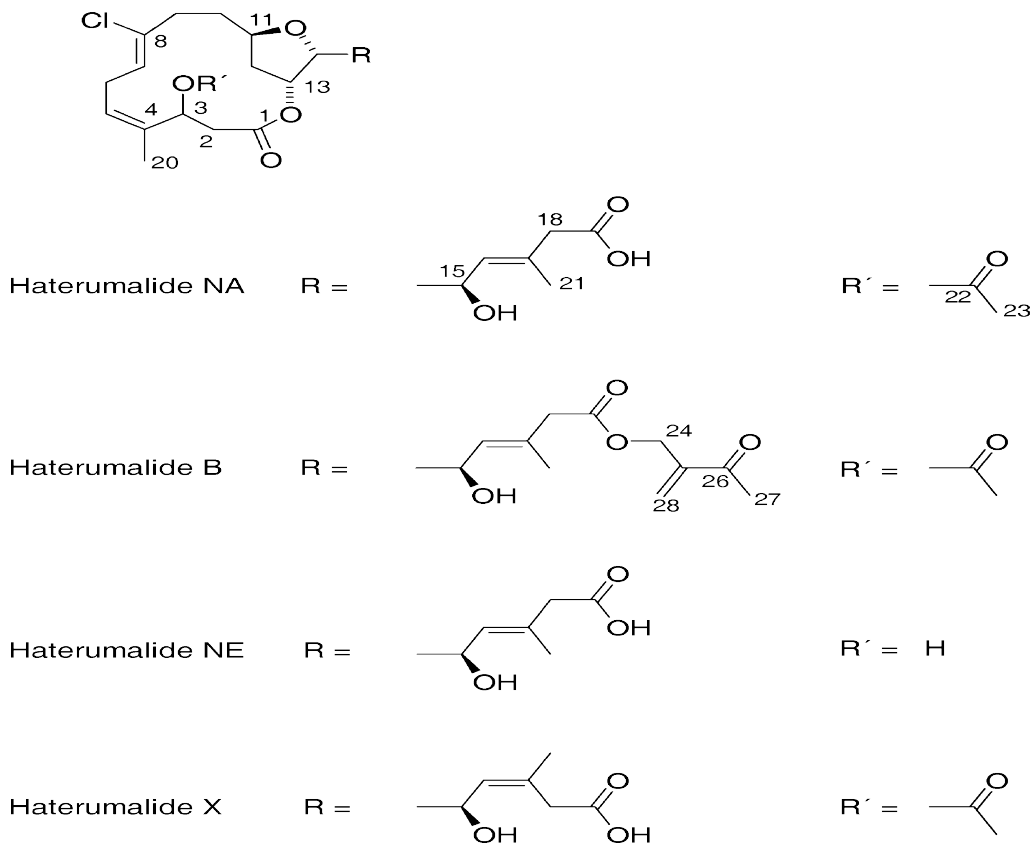
Recently, Levenfors et al. (2004), isolated several inhibitory compounds from the soil bacterium *Serratia plymuthica* A 153. The compounds showed inhibitory activity against the plant pathogenic fungus *Sclerotinia sclerotiorum* and were identified as chlorinated macrolides, haterumalide (NA, B, NE and X), 1-acetyl-7-chloro-1-H- indole and pyrrolnitrin.

More recently, Shen et al. (2007), evaluated the potential biocontrol properties of the biocontrol producer *Serratia plymuthica* A21-4 against the *Phytophthora capsici* which causes Phytophthora blight of pepper. The chemical structure of the antifungal compound was found to be identical to chlorinated macrolide. The chemical structures of antifungal antibiotics produced by terrestrial isolated *S. plymuthica* strains are shown in Figure 1.2. However, further research considering the biosynthetic pathway and genetic origin of haterumalide antibiotics produced by *S. plymuthica* is needed to confirm the involvement of a type I polyketide synthase cluster in the biosynthesis of these antibiotics (Vleesschauwer and Hofte, 2007).

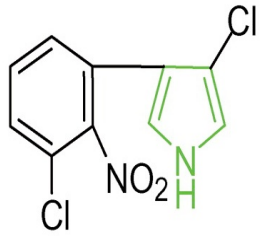
Some strains of terrestrial *S. plymuthica* isolates have been reported to produce red pigmentation associated with the antibiotic prodigiosin (red-pigmented tripyrrole antibiotic). Alström and Gerhardson (1987), isolated red-pigmented *Serratia plymuthica* G15 from plant rhizospheres, which showed antagonistic activity against the growth of fungal pathogens: *Gerlachia nivalis*, *Botrytis cinerea Pythium* spp, *Fusarium culmorum* and *Rhizoctonia solani*.

Kalbe et al. (1996), suggested that antifungal activity of the rhizosphere isolated *Serratia plymuthica* strains against the tested phytopathogenic fungi might have resulted from the production of prodigiosin, pyrrolnitrin and lytic enzymes. Berg (2000), reported that 21

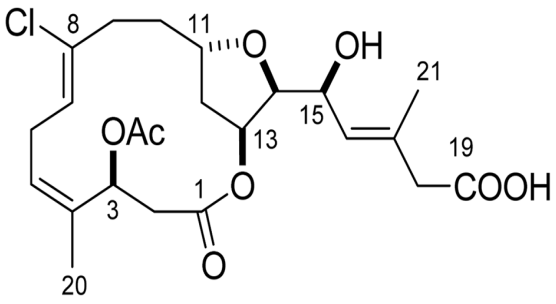
terrestrial *S. plymuthica* isolates produced antifungal antibiotics against the tested plant fungal pathogens; six of them were produced red pigmentation associated with the antifungal antibiotic prodigiosin.



1. Haterumalides.



2. Pyrrolnitrin (pyrrole rings highlighted in green).



3. Chlorinated macrolide (Macrocyclic lactone A21-4).

Figure 1.2: The chemical structures of some antimicrobial substrates produced by *S. plymuthica*: Haterumalide, Pyrrolnitrin and Macrocyclic lactone A21-4. From (Levenfors *et al*. 2004; Shen *et al*. 2007; Kirner *et al*. 1998).

1.6.3.2 Chitinase

Chitinases are the enzymes that hydrolysis chitin which is a universal element of most cell walls of fungi. This enzyme can be classified into two major groups: endochitinases which randomly cleave the chitin at internal sites, producing low molecular mass multimers of chitin, (i.e. chitotriose, chitotetraose, and diacetylchitobiose); and the exochitinases which are divided into two subgroups: N-acetyl-β-(1,4)-D-glucosaminidases and chitobiosidases (Patil *et al*., 2000). Based on these systems of classification, several kinds of chitinases have been identified in antifungal producer *S. plymuthica* strains. Several pieces of research have studied the role of chitinases in biocontrol activity (as antifungals). Frankowski et al. (1998), suggested that chitinases produced by *S. plymuthica* strains played an important role in antifungal activity and Kamensky et al. (2003), reported that *S. plymuthica* IC1270 secreted two exochitinases (N-acetyl-b-D-glucosaminidases) with a molecular mass of 67 and 89 kDa, and two endochitinase (chitobiosidases) with a molecular mass of 50 and 59 kDa.
Moreover, *S. plymuthica* strains (HRO-C48 and IC14), have been reported to produce an endochitinase N-acetyl-β-1, 4-D- hexosaminidase or chitobiase with a molecular mass of 100 kDa (Frankowski *et al*., 2001; Kamensky *et al*., 2003).

1.6.3.4 Siderophores

Iron is an important element supporting the growth of all organisms. It is essential for biological processes such as DNA synthesis and respiration. Despite iron being one of the abundant elements on our planet, the bioavailability of this element is limited, because of the very low solubility of ferric ions in several habitats, such as the sea, soil and plant surfaces (Höfte *et al*., 1993). Iron found in nature in mineral phases accumulates as iron hydroxides and oxide, and cannot directly be used by organisms (Höfte *et al*., 1993; Kraemer, 2004).

Under iron-limitation, bacteria release a range of low-molecular-weight substrates (iron chelators or siderophores) to transfer ferric irons from insoluble phases (minerals) to a soluble phase that can be utilised by organisms. Several of these siderophores are non-ribosomal peptides (NRPs) (Miethke and Marahiel, 2007). As biocontrol and

antimicrobial agents, these bacterial iron carriers (siderophores) reduce the iron available in the rhizosphere, thus preventing soil-borne pathogens such as plant pathogenic fungi from developing and subsequently inhibiting their growth (O'Sullivan and O'Gara, 1992; Loper and Henkels, 1999).

Several studies have reported that terrestrial *S. plymuthica* isolates, including strains such as IC14, IC1270, HRO-C48 and 3Re4-18, have been shown to produce potential siderophore molecules with antifungal activity when grown on free-iron media (Kalbe *et al*., 1996; Frankowski *et al*., 1998; Kamensky *et al*., 2003; Ovadis *et al*., 2004; Berg *et al*., 2005).

1.7 Polyketides (PKs) and Non-ribosomal peptides (NRPs)

Non-ribosomal peptides and polyketides are two groups of complex natural products, which are found in plants and micro-organisms. These metabolites result from multi-modular enzyme systems called non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) and have various structures. For instance, they can be linear like ribosomally synthesised peptides, branched, cyclic (totally or partially) or polycyclic (Minowa *et al*., 2007). The various structures of these peptides allows them to engage in a wide range of important biological and pharmacological activities; including antibiotics, such as vancomycin (a non-ribosomal peptide antibiotic), erythromycin (a polyketide antibiotic), amphotericin (a macrolide) and yersiniabactin (a siderophore) (Pfeifer *et al*., 2003). The chemical structures of some of these PKs and NRPs compounds are shown in Figure 1.3.

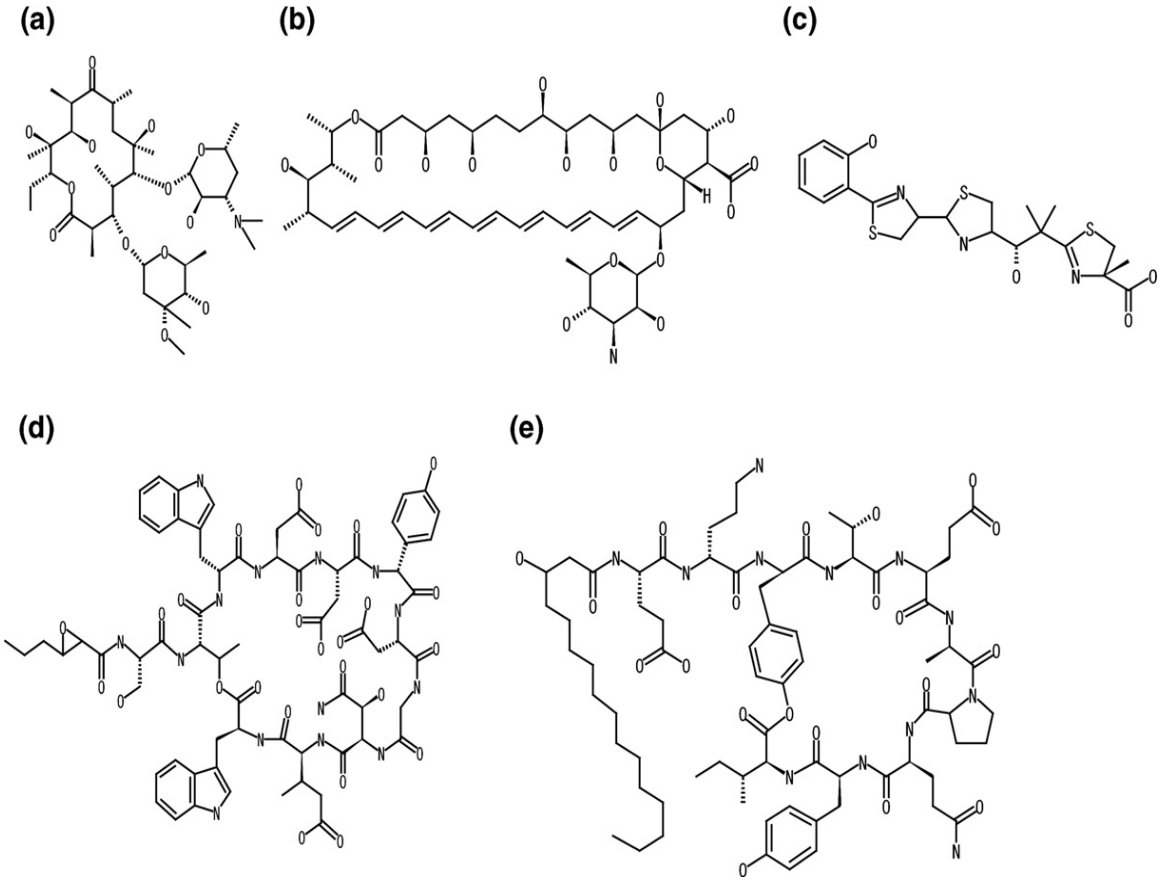


Figure 1.3: The chemical structures of polyketide and non-ribosomal peptide compounds (a) Erythromycin; (b) amphotericin; (c) yersiniabactin; (d) CDA; and (e) fengycin (lipopeptide antibiotics). From Minowa *et al.*(2007).

1.7.1 PKs and NRPs produced by the terrestrial isolates of *S. plymuthica*

The first report of polyketide compounds to be produced from *S. plymuthica* strains was in 2001 by Thaning et al. who isolated *S. plymuthica* from soil *and* produced a bioactive compound that inhibited the fungal plant pathogen *Sclerotinia sclerotiorum*. The compound was identified as a chlorinated macrolide and termed haterumalide NA. Three years later, Levenfors et al. (2004) isolated several inhibitory compounds from different soil sourced *S. plymuthica* (strain A153) including polyketide compounds (chlorinated macrolides and haterumalide (NA, B, NE and X). More recently, Shen et al. (2007) identified a potential polyketide compound with antifungal activity which was found to be a chlorinated macrolide (macrocyclic lactone A21-4). An examination of the *Serratia*

genus in general (e.g. *Serratia* sp. ATCC 39006 and *S. marcescens* ATCC 274) characterising the prodigiosin gene cluster (*pig* cluster), and the result showed that the sequenced *pig* gene cluster contains genes with some similarity to non-ribosomal peptide synthases (NRPSs), polyketide synthases (PKSs) and the red proteins of *Streptomyces coelicolor* (Harris *et al*., 2004).

In contrast, some studies have indicated that the similarities of the structure of the haterumalide biosynthesis genes in macro-organisms such as seaweed suggests that the biosynthetic pathway of the haterumalides involve a type I polyketide synthase gene cluster, similar to the haterumalide biosynthesis in bacteria, such as that found in *Pseudomonas* sp. (Rangaswamy *et al*., 1998; Nowak-Thompson *et al*., 1999). Nevertheless, further research considering the biosynthetic pathway and the genetic origin of haterumalide antibiotics produced by *S. plymuthica* is necessary to confirm the involvement of the type I polyketide synthase gene cluster in the biosynthesis of these antibiotics (Vleesschauwer and Hofte, 2007).

1.8 Quorum sensing (QS)

Quorum-sensing systems (QS), in bacterial cells, are regulatory mechanisms for controlling gene expression, via the production of small diffusible chemical signal molecules (autoinducers). The autoinducers express or inhibit certain gene(s) in response to bacterial cell population density (Miller, 2001). The increase of the bacterial cell-population density results in increases in autoinducer concentration, and therefore, increasing levels of these signalling molecules to be sensed allowing surrounding bacteria to respond correspondingly. Gram-negative and gram-positive bacteria use quorum-sensing systems to regulate several physiological activities including virulence, competence, symbiosis, conjugation, motility, sporulation, biofilm formation and antibiotic production. In general, gram-positive bacteria use oligopeptides as autoinducers, whereas gram-negative bacteria use *N*-acyl-$_{L}$-homoserine lactones (AHLs), which have been very well studied on this group of bacteria (Figure 1.4) (Miller, 2001; Waters and Bassler, 2005; Van Houdt *et al*., 2006; Van Houdt *et al*., 2007a). Although the nature of autoinducers, the target genes controlled and the mechanisms of quorum sensing systems differ, in every situation the ability to communicate with one another allows bacteria to control the gene expression, and thus the behaviour, of the whole

community. This QS mechanism impart upon bacterial communities some of the qualities of eukaryotic organisms (Miller, 2001).

Studies have suggested that the quorum-sensing system plays a role in the expression of specific genes, including in some instances those for antimicrobial production (Labbate *et al*,. 2004). Antimicrobial production in bacteria has been reported to be controlled globally by Quorum sensing and two-component systems. Quorum sensing systems release autoinducers such as AHLs, which bind to transcriptional regulators, which in turn bind to the promoters of the targeted gene(s) to control gene expression. However, low levels of autoinducers (AHL) have an opposite effect. The two component systems allow organisms to control the internal environment with regards to exterior factors (Moons *et al*., 2006; Van Houdt *et al*., 2007a; Van Houdt *et al*., 2007b).

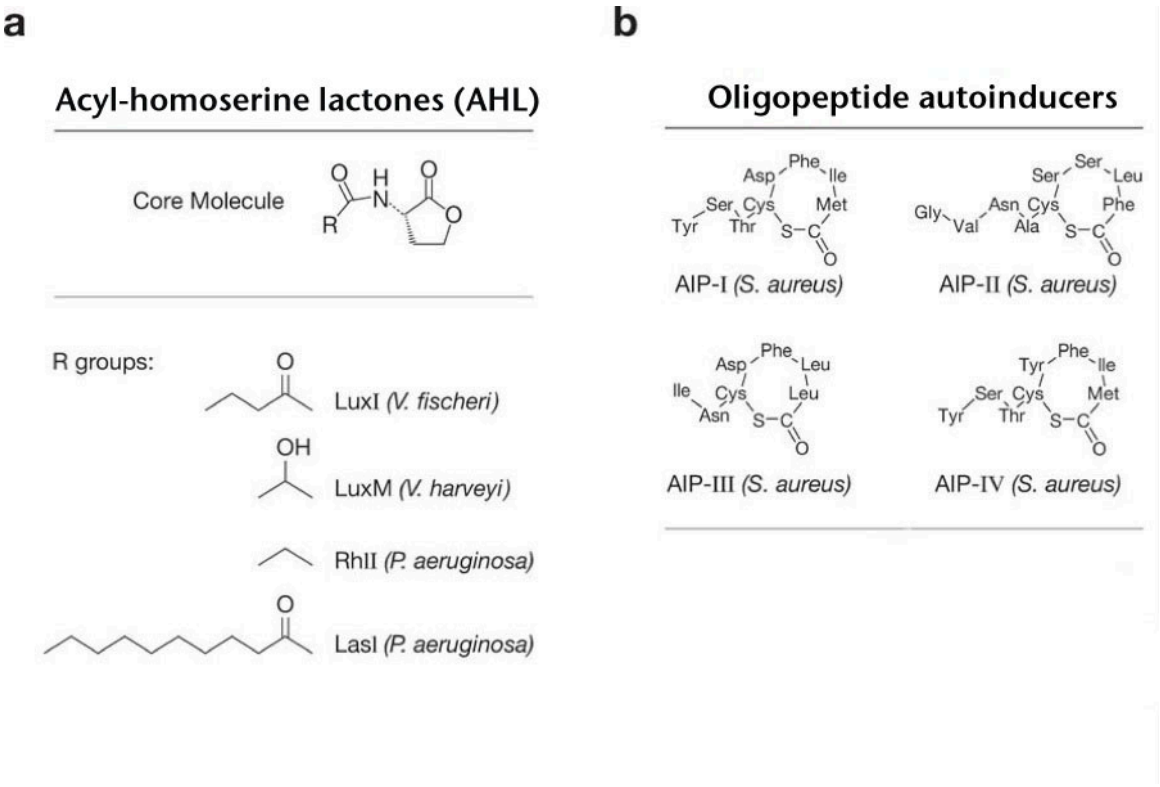


Figure 1.4: The chemical structure of the most intensively studied autoinducer system in gram-negative bacteria (a) and gram-positive bacteria (b). From Waters and Bassler (2005).

1.8.1 Quorum sensing and antimicrobial production in *S. plymuthica*

In the terrestrial *S. plymuthica* isolates, different quorum sensing and target genes have been studied. ALH-dependent quorum-sensing signalling has been linked with several phenotypes; for instance, the production of chitinase, nuclease, protease, antibacterial compounds and biofilm fermentation (Van Houdt *et al*., 2007a).

The first report of the role of the QS system in regulating the production of an antimicrobial in *S. plymuthica* was a study looking at the production of pyrrolnitrin (Liu *et al*., 2007). The study showed that *S. plymuthica* HRO-C48 (rhizosphere isolated), produced several quorum-sensing signalling molecules (AHLs) which included; N-hexanoyl-HSL, N-butanoyl-HSL and N-3-oxo-hexanoyl-HSL (OHHL), which are required for the production of pyrrolnitrin.

Moreover, a study performed by Pang et al. (2008), confirmed the involvement of QS in the antimicrobial production *S. plymuthica* HRO-C48 (Pang *et al*., 2008). Also, Van Houdt et al. (2007b), reported that the production of an antimicrobial compound by *S. plymuthica* RVH1 was quorum sensing controlled and indicated that the LuxR and SplR, homologs of *S. plymuthica* RVH1 were repressors of AHL genes. Some examples of the autoinducing molecules (AHLs) identified in *S. plymuthica* strains are shown in Table 1.5

Table 1.5 Examples of quorum sensing systems identified in *S. plymuthica* strains.

***S. plymuthica* strains**	**AHLs.**	**LuxI/LuxR.**	**Reference.**
IC1270 3	3-hydroxy-C6-HSL, 3-hydroxy-C8-HSL	SplI/SplR	Ovadis *et al*. (2004).
RVH1	C4-HSLw, C6-HSLw 3-oxo-C6-HSL	SplI/SplR	Van Houdt *et al*. (2007b)

1.9 Aims of this study

The research reported within this study, had five aims:

1) The characterisation of an antimicrobial producer bacterium isolated from the surface of seaweed (*Ascophyllum nodosum*).
2) The investigation of antimicrobial activity against a wide variety of indicator micro- organisms; such as food borne pathogens, food spoilage micro-organisms and medically important pathogenic micro-organisms.
3) The study and modification of cultivation methods, to induce the production of antimicrobial compound(s) in a liquid culture.
4) The characterisation of the antimicrobial compound(s) produced by the marine bacterium.
5) Identification of the gene(s) responsible for antimicrobial production.

MATERIALS AND METHODS

2. Materials and Methods

2.1 General Methods

All chemicals, media, reagents, enzymes, buffers and other materials were supplied by Thermo Fisher Scientific (UK) or Sigma-Aldrich (UK). All the materials in this study were handled and stored according to the manufacturer's instructions. Centrifugation was carried out using a microfuge for small samples, using 1.5ml microcentrifuge tubes. Large volume samples up to 1 litter, however, were centrifuged using a large centrifuge (Beckman Avanti J-26 XP). All centrifugations were run at an appropriate speed and at either room temperature or 4 ^{o}C. In this study, solutions and glassware were autoclaved for 15 min at 121 ^{o}C.

2.1.1 Microbial strains used in the study

2.1.1.1 Marine microorganisms

From the screening of seaweed associated marine microorganisms, for their ability to produce antimicrobial compounds, (samples collected from Aberdour beach, Scotland on the 17th of November 2009), the laboratory observed that a red-pigmented micro-organism inhibited the growth of a filamentous microorganism (Figure 2.1). This particular antimicrobial producer was the subject of this study (termed P) and the filamentous micro-organism whose growth was inhibited (sensitive strain) was termed F.

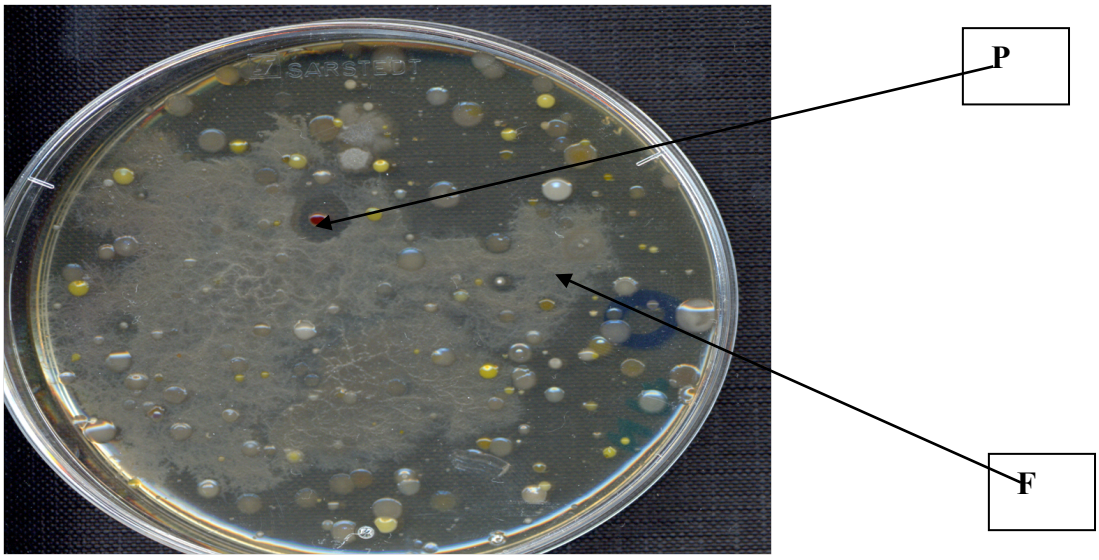


Figure 2.1 Initial nutrient agar plate with unknown colony (P), inhibiting the growth of filamentous microorganism (F).

2.1.1.2 Test microorganism strains

The sensitive strains used in this study, for antimicrobial screening represent certain concerns for the medical sector, such as: multi-drug resistant human pathogenic bacteria, pathogenic yeast, food spoilage and food borne pathogenic micro-organisms. Table 2.1 shows the microorganisms, used for this study. They were obtained from the School of Life Sciences Heriot Watt University.

Table 2.1 Micro-organisms used in this study

Microorganisms /Lab ID	**Source**	**Category**
Serritia plymuthica (P)	D. Jamieson[1]	Antimicrobial producer
Serratia marcescens (ATCC13880).	P. Cyphus[2]	Antimicrobial producer
Bacillus cereus var mycoides (F)	D. Jamieson[1]	Test strain
Serratia plymuthica DSM 4540	DSMZ	Reference strain
Methicillin resistant *Staphylococcus aureus* (MRSA)	H. Rahman[3]	Test strain
Staph. epidermidis	H. Rahman[3]	Test strain
Vancomycin resistant *enterococci (VRE)*	H. Rahman[3]	Test strain
Listeria monocytogenes scott A	W. Mitchell[4]	Test strain
L. monocytogenes NCTC7973	W. Mitchell[4]	Test strain
Clostridium difficile	W. Mitchell[4]	Test strain
Candida albicans (NCTC3153)	D. Jamieson[1]	Test strain
Candida albicans (NCTC5314)	D. Jamieson[1]	Test strain
Pichia angusta	D. Jamieson[1]	Test strain
Penicillium expansum	P. Cyphus[2]	Test strain
Pseudomonas aeruginosa	P. Cyphus[2]	Test strain
E. coli BW20767	P. Morris[5]	Test strain
E. coli D28	S. Dewar[6]	Test strain
E. coli	P. Cyphus[2]	Test strain

Legend to Table

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2 P. Cyphus, School of Life Sciences, Heriot Watt University, Edinburgh, UK
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2.1.1.3 Established bacterial strains used in the study

Table 2.2 Established bacterial strains used in the study

Strains	**Genotype/Characteristic**	**Source/ Reference**
E. coli BW20767	*leu-63*::IS*10 recA1 zbf-5 creB510 hsdR17 endA1 thi uidA*(MluI::*pir*) chromosome::RP4-2 Tc::Mu Km::Tn*7*; Tpr Smr. Transposon donor strain. (LB+ Kan [50 µg /ml])	Larsen *et al*. (2002)
E. coli S17-1/λ *pir*	*pro thi hsdR recA* chromosome::RP4-2 Tc::Mu Km::Tn*7*/*_pir*; Tpr Smr. Maintaining Transposon junction plasmids. (LB+ Str[20 µg /ml])	Larsen *et al*. (2002)
E. coli XL1-Blue	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacIqZΔM15* Tn*10* (Tet^r)].	Stratagene, UK
Chromobacterium violaceum (CV0blu)	Triple mini-Tn*5* mutant derived from *C. violaceum* ATCC 31532. N-acylhomoserine lactones (AHLs) biosensor Hgr*cviI::*Tn*5xylE* Km^r Cm^r, plus spontaneous Sm^r and another uncharacterised mutation allowing a stronger response to AHLs	Swift *et al*. (1997)
Chromobacterium violaceum (CV031)	Mini-Tn5 mutated derivative of *Chromobacterium violaceum* ATCC 31532 which was originally selected for overproduction of the purple violacein pigment.	M. K. Winson, pers. Comm, Heriot Watt University

2.1.1.4 Bacterial strains created during this study

Table 2.3 Bacterial strains created during this study

Strains	**Description**	**Markers**	**Source**
M4	Non antimicrobial producer mutant no 4, Tn*5*-RL27 (Km^R-*ori*R6 K) delivery vector: inserted into the chromosomal DNA of *S. plymuthica* (P)	Km^R, Ap^R	This study
M5	,, ,, mutant no 5 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M6	,, ,, mutant no 6 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M7	,, ,, mutant no 7 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M8	,, ,, mutant no 8 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M9	,, ,, mutant no 9 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M10	,, ,, mutant no 10 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M11	,, ,, mutant no 11 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M12	,, ,, mutant no 12 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M13	,, ,, mutant no 13 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M14	,, ,, mutant no 14 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M15	,, ,, mutant no 15 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M16	,, ,, mutant no 16 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study
M19	,, ,, mutant no 19 ,, ,, ,, ,, ,, ,,	Km^R, Ap^R	This study

2.1.1.5 Established plasmids and constructed plasmids used in this study

Table 2.4 Established plasmids and constructed plasmids used in this study

Plasmid	Size/bp	Description and/or construction	Markers	Source
pRL27	4080	Tn*5*-RL27 (Km^R-*ori*R6 K) delivery vector: circularised PCR fragment from pRL23 (primers *tetA*p-for and *oriT*-rev)	Km^R	Larsen *et al.* (2002)
pBluescript II KS(-)	2961	DNA cloning victor	Ap^R	Stratagene, UK
TJP4	˜8000	Transposon junction plasmid No 4 carrying Tn*5*- insertion in P chromosomal DNA from strain PM4 on a *Bam*H1 fragment self ligated with T4 DNA ligase, and transformed into *E. coli* S17-1/λ *pir* strain	Km^R, St^R	This study
TJP5	˜5000	Transposon junction plasmid No 5 carrying the Tn*5*- insertion in P chromosomal DNA from strain PM5 on a *Bam*H1 fragment, self ligated with T4 DNA ligase and transformed into *E. coli* S17-1/λ *pir* strain	Km^R, St^R	This study
TJP7	˜8000	Transposon junction plasmid No 7 carrying Tn*5*- insertion in P chromosomal DNA from strain PM7 on a *Bam*H1 fragment, self ligated with T4 DNA ligase and transformed into *E. coli* S17-1/λ *pir* strain	Km^R, St^R	This study
TJP8	˜6000	Transposon junction plasmid No 8 carrying Tn*5*- insertion in P chromosomal DNA from strain PM8 on a *Bam*H1 fragment, self ligated with T4 DNA ligase and transformed into *E. coli* S17-1/λ *pir* strain	Km^R, St^R	This study

2.1.1.6 Maintenance of microbial cultures

Microbial stock cultures were prepared for all the micro-organism strains within the study, using ProTec bacterial preservers (provided by Technical Service Consultancy limited). Stock cultures were then stored at –70 ^{o}C. Working stocks of microbial cultures were maintained on NA plates, stored at 4 ^{o}C.

2.1.2 Antibiotics used in the study

Antibiotic	**Stock concentration* (Mg/ml)**	**Final concentration in solid and liquid media (µg/ml)**
Kanamycin (Km)	50	50
Ampicillin (Ap)	50	50
Streptomycin (St)	50	20
Tetracycline (Tet)	50	50

*Stock solution prepared in distilled water and filter sterilised.

2.1.3 Bacterial growth, media and culture conditions

Microbial cultures were regularly grown on nutrient agar (NA) plates or in nutrient broth (NB, 5g/L Sodium Chloride) with incubation at 21 ^{o}C for 18-24 hrs. Additional media was also used such as: Marine broth (MB, 19.45 g/L Sodium Chloride) DNase agar, Tryptone soya agar, Tryptone soya broth, Brain heart infusion agar, Brain heart infusion broth and Blood agar, Gelatin agar (NA plus 8% Gelatin w/v), Casein agar (NA plus 2% Skimmed milk w/v) and Maximum Recovery Diluent (MRD, Peptone Saline Diluent (1.0 g/L Peptone and 8.5 g/L Sodium Chloride). *E. coli* strains, however, were grown on Luria-Bertani (LB, 10g/L Sodium Chloride) broth or agar. For cultures, purified single colonies of bacterial strains were grown in nutrient broth (NB) or LB broth at 37 ^{o}C for 18-24 hrs. Media component were obtained from Oxoid Ltd. The medium was prepared

and autoclaved, once it had cooled enough to be handled (~55 ^{o}C) any (filter-sterilised) antibiotics required, were then added to the appropriate concentration.

2.1.4 Cultivation conditions

Growth was either on agar plates or in shake flask cultures. Shake flask cultures were incubated aerobically at 21 ^{o}C with shaking (220 r.p.m.) for up to 24hrs.

2.1.5 Spot test and disc diffusion assays for antimicrobial activity

Antimicrobial activity was performed using two methods; the disc diffusion test (Mearns-Spragg *et al*,. 1998) and also the spot test against various of test micro-organisms (Table 2.1). Briefly, a fresh overnight culture of the appropriate sensitive strain (100 µl) was spread onto an NA plate using a metal spreader. Then, the inoculated plates were left to dry before spotting a 10 µl volume of a fresh overnight culture of the test microorganism directly onto the surface of the inoculated plates (Spot test).

A disc diffusion assay was performed using Whatman 6 mm paper discs, saturated with 100 µl of test samples. Briefly, a 20 µl volume of liquid samples was dropped directly onto the surface of the disc. Then, the saturated discs were left on the bench for up to 1hr to dry before addition of another 20 µl volume of the test liquid. Following this, the discs were placed onto the inoculated nutrient agar plates with the indicator micro-organisms.

The tests (spot test and disc diffusion) were carried out in triplicate and the plates incubated either aerobically or / anaerobically, at appropriate temperatures (typically 21, 30 and 37 ^{o}C) for up to 24 hrs to 5 days, depending on the test strains. The inhibition zone was measured edge to edge (spot test) or edge the disc (disc diffusion assay), in mm and results recorded according to the presence (or absence) of a clearing zone.

2.1.6 Gram reaction, colony morphology and microscopy

An overnight fresh culture of the test micro-organism, was subjected to Gram staining using the Hucker and Conn method (Hucker and Conn, 1923), then examined with a light microscope at x1000 magnification, using an oil immersion lens. Colony morphology on the surface of agar plates was also examined, using the naked eye.

2.1.7 Sensitivity tests

Antibiotic sensitivity testing was performed using antibiotic sensitivity discs (Abtek Biological Ltd). The test was conducted on NA plates, and the plates incubated aerobically at 21 ^{o}C for up to 24 hrs. The result was recorded by measuring the clearing zone around each antibiotic test disc.

2.1.8 Total Viable cell count

The total viable count for microbial growth samples was carried out in this study. Briefly, one ml of overnight culture was diluted with 9 ml of 0.1% (w/v) sterile maximum recovery diluent (MRD, Section 2.1.3) to obtain a 10^{-1} dilution. Serial dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) from the 10^{-1} dilution were prepared, and 0.1 ml volumes of the 10^{-1} 10^{-4}, $10^{-5}$and 10^{-6} of the diluted sample were then surface plated (spread plate method), in duplicate, onto NA plates agar, which were aerobically incubated at 21 ^{o}C for up to 24 hrs. The colony forming units (CFU) were counted, following the incubation period and the results were recorded as log 10 of colony forming unit CFU /ml in the plates containing between 25–250 colonies.

2.2 DNA techniques

2.2.1 Extraction of genomic DNA

Pure cultures were obtained from the frozen stock culture by directly plating out on an agar plate (NA or LB with / without the appropriate antibiotic). Briefly, a colony was then scratched up from frozen stock with a sterile wooden stick and streaked out onto an agar plate. Following the incubation period (dependent on the growth conditions of each test microorganism) a single colony from the streaked plate was placed into a 10ml flask of growth media. Following appropriate incubation conditions, saturated cultures were transferred to sterile 1ml centrifuge tubes and the cells pelleted using centrifugation, to harvest the microbial cells. Briefly, microbial cultures were spun in 1.5 ml Eppendorf tubes at 13.000 rpm for 1 min in a bench top microcentrifuge. The supernatant was then discarded and the genomic DNA from the bacterial cells (pellets) was extracted using a DNeasy® Tissue kit (Qiagen) and following to the manufacturer's instructions. All the DNA samples in this study were stored at 4 ^{o}C.

2.2.2 Extraction of plasmid DNA

In order to prepare bacterial cells for plasmid DNA purification, *E. coli* cells (Section 2.1.1.5) were treated as discussed, in the previous Section (2.2.1). Plasmid DNA was extracted from *E. coli* cells by the QIAprep spin miniprep Kit (QIAprep® Miniprep, Qiagen) using a microcentrifuge method, according to the manufacturer's instructions. Plasmid DNA samples were stored at 4 ^{o}C until required.

2.2.3 Concentration of DNA

The method described below was used to purify and/or concentrate DNA from aqueous solutions. One volume of DNA solution was mixed with 0.1 volume of 3 M Sodium Acetate, pH 5.2 and two volumes of Ethanol (96%). The DNA was allowed to precipitate at -20 ^{o}C for 30 minutes, followed by centrifugation at 13000 rpm for 10 minutes. The

supernatant was then discarded and the pellet washed by adding 1ml Ethanol (70%). A further centrifugation was carried out (13000 rpm for 10 minutes), the supernatant was discarded and the pellet dried at 37 ^{o}C for 1 hr. The DNA was then suspended in an appropriate volume of sterile dH_2O and stored at 4 ^{o}C.

2.2.4 Restriction enzyme digestion of DNA

Chromosomal DNA or plasmid DNA was cleaved, using restriction endonucleases. The digestion sample contained DNA (1-2 µg, plasmid DNA or up to 10 µg, genomic DNA) the appropriate restriction enzyme(s), an enzyme specific buffer, and sterile dH_2O to bring the digestion sample to its final volume. All restriction enzymes and buffers utilised in the study were obtained from MBI Fermentas. The digestion samples were incubated at 37 ^{o}C for up to 24 hrs (plasmid DNA digest or genomic DNA digests). Following incubation, the digestion samples were spun for 5 seconds and then placed in an incubator at 80 ^{o}C for 20 minutes, for enzyme thermal inactivation, (as recommended by the manufacturers). The sample was immediately placed on ice for 10 minutes and the efficiency of the DNA digestion reaction was evaluated using gel electrophoresis (Section 2.2.8).

2.2.5 DNA ligation

Up to 20µl of a digested DNA sample (Section 2.2.4) was subjected to a ligation reaction, within a total volume of 40µl. The ligation (40µl) contained 20µl of digested DNA, 1µl of T4 DNA ligase (5 units/µl; Fermentas), 5µl of 10x T4 DNA ligase buffer and 14µl sdH_2O. The ligation mixture was then incubated at room temperature for 24hrs. Following the period of incubation, the mixture was incubated at 70 ^{o}C for 10 minutes to inactivate the ligase (as recommended by the manufacturers). The mixture was then immediately placed on ice for 10 minutes. DNA ligation reaction efficiency was evaluated using gel electrophoresis (Section 2.2.8).

2.2.6 The Polymerase Chain Reaction (PCR)

PCR reagents were stored at -20 ^{o}C and mixed in 0.5ml Eppendorf tubes (Sarstedt PLC). To ensure careful application, a fresh pipette tip and sdH_2O was used for each addition. All PCR reactions were conducted in a total volume of 50 µ1. PCR controls were also prepared using sdH_2O instead of the template DNA. The PCR machine used was a Techne progene thermocycler. The standard protocol used in PCR experiments is detailed below in Table 2.5 and Table 2.6. Long PCR reactions were also carried out using a Long PCR kit (Long PCR Enzyme Mix, Fermentas), according to the manufacturer's instructions (> 30 Kb). All primers used in this study were supplied by MWG Eurofinns, and were re-suspended in sdH_2O to a final concentration of 100 pmol/µl before storing at -20 ^{o}C. (they are listed in Table 2.7).

Table 2.5 PCR conditions

Temperature	**Time**	**Number of cycles**	**Event**
95 ^{o}C	5 mins	1	Initial denaturation
95 ^{o}C 56 ^{o}C 72 ^{o}C	1 mins 1 min 2 mins	25-35	Denaturation Annealing Elongation

Table 2.6 PCR components

PCR recipe	**Volume of stock solution / (50µl)**
DNA template	5µl
Forward primer (10 pmol)	1µl
Reverse primer (10 pmol)	1µl
$MgCl_2$ (50mM)	5µl
TAQ Mix*	10µl
Milipore (Mili-Q) H_2O	27µl
Taq DNA polymerase	1µl

*TAQ Mix for PCR reaction

Recipes	**Quantity Per 1000µl**
dATP (100mM)	10 µl
dTTP (100mM)	10 µl
dGTP (100mM)	10 µl
dCTP (100mM)	10 µl
Milipore (Mili-Q) H_2O	460 µl
10_X NH_4 buffer	500µl

Table 2.7 List of Primers used in this study

Primer name	**Sequence**	**Target**	**Reference**
27 F	AGAGTTTGATCMTGGCTC	16S rRNA gene	Lane (1991)
1492R	TACGGYTACCTTGTTACG ACTT	16S rRNA gene	Lane (1991)
AGPT-F	ATTCAACGGGAAACGTCT TG	Km^R gene	Callum Scott Thesis, Heriot Watt University
AGPT-R	ACTGAATCCGGTGAGAAT GG	Km^R gene	Cullum Scott Thesis, Heriot Watt University
tpnRL17–1	AACAAGCCAGGGATGTA ACG	Outward-directed prime (Anneals to the *oriR6K* of the transposon)	Larsen *et al.* (2002)
tpnRL13–2	CAGCAACACCTTCTTCAC GA	Outward-directed prime (Anneals to the end of Km^R gene)	Larsen *et al.* (2002)
ox 495	CGAACCTGTGGCTCCAAA GG	Anneals to a segment of the *S. plymuthica* genomic DNA present in TJP7	This study
ox 496	GGTGCTAATGCAGCACTT GGA	As for ox 495 TJP 7	This study
ox 497	ACTGCCTGTTCGTCCTCTT TAG	As for ox 495 TJP 5	This study
ox 498	TCCAGGTGTTCAAGCAAG CGC	As for ox 495 TJP 5	This study
ox 501	CAGTCATTGCTGAAGAGC AGG	As for ox 495 TJP 7	This study

Table 2.7 continued

ox 502	ACACCAAAGGAACGGCTCGA	As for ox 495 TJP 7	This study
ox 503	GCTTGGACCGATAACGCTACC	As for ox 495 TJP 5	This study
ox 504	TCCGTATGGGGAAGAGGATT	As for ox 495 TJP 5	This study
ox 505	CCAATTGGACATACACCCATGTC	As for ox 495 TJP 7	This study
ox 506	GACCGGAACTCACGATAGAGC	As for ox 495 TJP 7	This study
ox 507	GAAGCGTAGACTGACGCAACT	As for ox 495 TJP 5	This study
ox 508	CTCTGATTGACGGTAGTGGAGTG	As for ox 495 TJP 5	This study
ox 511	CATGATCCAGTTCACAGTGTC	As for ox 495 TJP 8	This study
ox 512	AATTGCCGTCTTATGAGAGGCC	As for ox 495 TJP 8	This study
ox 513	TGTTATCTGGTGCTTGCCTTG	As for ox 495 TJP 7	This study
ox 514	CTTGCGGGTCGATACTCC	As for ox 495 TJP 7	This study
ox 517	AAAGGATCCATAAACTCAGCTTCGCG	Anneals to portions of the *S. plymuthica* genomic DNA present in TJP 7 *Bam*H1 site in red)	This study
ox 518	AAAGGATCCTAAAACACCAAAGGAACGG	As for ox 517 TJP 7	This study
ox 519	AAAGGATCCATCCACTCAGCTTCTTG	As for ox 517 TJP 5	This study

Table 2.7 continued

ox 520	AAAGGATCCGCAAGCTCT GGTATTG	As for ox 517 TJP 5	This study
ox 521	AAAGGATCCTTATCGTAT TGGTTTAGTAGTG	As for ox 517 TJP 8	This study
ox 522	AAAGGATCCCATAAAAA GGGCGCTG	As for ox 517 TJP 8	This study

2.2.7 Ribotyping

The sequencing of the 16S rRNA gene was performed according to Charrier *et al*., (2006) with the primers as described by Lane (1991) and listed in Table 2.7. Genomic DNA for ribotyping was prepared as described in Section 2.2.1 and the 16S rRNA gene was amplified by PCR as details in Section 2.2.6. The amplified fragment of the rDNA gene was then cleaned using a using a QIA quick PCR Purification Kit (Qiagen) to remove residual template DNA nucleotides and primers prior to sending to the University of Edinburgh sequencing facility (Genepool). The resulting sequences were used to search the nucleotide sequence databases using a nucleotide query and the BLAST program (www.blast.ncbi.nlm.gov/blast.cgi).

2.2.8 DNA separation by gel electrophoresis

Agarose gels were prepared by dissolving agarose powder to a final concentration of 0.7 to 1% (w/v) in 1x TAE buffer (pH 8.0) and then melted using a microwave oven; after which the molten gel was left to cool down to about 50 ^{o}C and ethidium bromide (final concentration 0.15 µg/ml) was added and the gel then was cast and left to set. Before loading the DNA samples into the gel wells, 5µl of the DNA sample was added to 2µl of 5x gel loading buffer (0.25% bromophenol blue, 25% glycerol, 1% SDS, 150 mM EDTA) and mixed gently. A 1kb DNA ladder (MBI Fermentas) was used to size the DNA fragments and the gels were run in 1x TAE buffer at 95v for 55 minutes. After this, the electrophoresed DNA was visualised using ultra-violet light and a photograph was taken.

Tris Acetate-EDTA (50 x TAE, PH 8.8) buffer/ Litre:

Recipes	**Quantity Per 1000ml**
Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100 ml
Distilled water	Up to 1000 ml

2.2.9 DNA Sequencing of plasmid DNA

The DNA samples for sequencing were purified, when required, using a QIA quick PCR Purification Kit (Qiagen) with the microcentrifuge method, according to the manufacturer's instructions. All sequence samples were prepared in 8 strip tubes and covered with 8 strip caps (PCR.0208 C, AXY GEN® Scientific.USA). Briefly, 1µl of purified PCR product or 200-400ng purified plasmid DNA was added to 4µl of sdH_2O and 1µl of desired primer (3.2 pmol). The sequencing samples were sent to Edinburgh University for cycle sequencing. The generated DNA sequences were compared to the reference bacterial gene sequences available in nucleotide databases (Genbank), using a nucleotide query BLASTN and X.

2.2.10 Gene cloning

Chromosomal DNA was isolated, as discussed in Section 2.2.1, and subjected to long PCR reaction (Section 2.2.6) using appropriate primers. Next, the PCR product was cut with the restriction enzyme *BamH*1 (Section 2.2.4), and then purified using a QIA quick PCR Purification Kit (Qiagen) with the microcentrifuge method, according to the manufacturer's instructions. The pBluescript (pBS) plasmid was extracted from the *E. coli* pBluescript II KS (-)(Section 2.2.2) (For plasmid map see Figure 2.2), and cut with *BamH*1 as described in Section 2.2.4, and also purified. Next, the digested purified DNA samples (PCR product {insert} and pBS plasmid {vector}) were ligated together using T4

DNA ligase (Section 2.2.5) to create a recombinant plasmid cloning reaction, which was carried out using the following equation;

3: 1 molar ratio insert: vector used in ligation reaction as follow:

$$\text{Insert Mass in ng} = 3\times \left[\frac{Insert\ length\ in\ bp}{Vector\ lenght\ in\ bp}\right] \times VectorMass\ in\ ng$$

Following ligation, a 1-10µl-ligation mixture was transformed chemically into *E. coli* XL1-Blue competent cells, as mentioned in Section 2.2.13. Afterwards, screening for successful transformants was completed using blue/white colour screen. Briefly, the transformed cells were plated out on LB plates containing 50 µg/ml Ap and 40µl of 0.1 M Isopropyl thioglactoside (IPTG) and 2% 5-bromo-4-indolyl-beta –D- galactopyranoside (Xgal) that was spread on top of the LB plates and left to dry before plating, plates were incubated aerobically at 37 ^{o}C for 18-24hrs. Following the period of incubation, the white colonies (transformants) were selected randomly and sub-cultured with the selective media (LB+AP) and then subjected to mini plasmid preparations as described in Section 2.2.2.

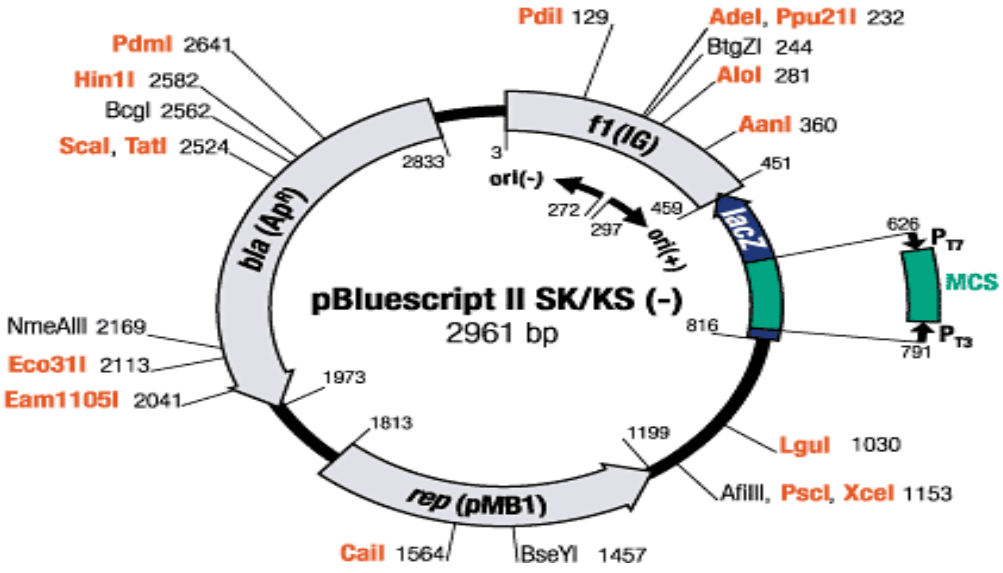


Figure 2.2 Graphic map (circular) of the cloning vector pBluescript (pBS) plasmid. From Thermo Scientific.

2.2.11 Random priming

Generation of digoxigin-labelled probes using random priming was as follows; plasmid DNA was extracted (Section 2.2.2), and subjected to a PCR reaction (Section 2.2.6), using specific primers (Table 2.7). The PCR product was purified using PCR purification Kit (50, Qiagen) according to the manufacturer's instructions. The purified DNA was labelled with digoxigenin-11dUTP (DIG), according to the manufacturer's instructions, using a DNA labelling kit (Boehringer Mammheim). The probe was stored at -20 ^{o}C until required.

2.2.12 Southern blot hybridisation

Southern blotting was performed in the study, using capillary action, as described by Southern (1975). The bacterial genomic DNA and Plasmid DNA were extracted as described in Sections 2.2.1 and 2.2.2 respectively. Chromosomal DNA was digested with *BamH*I and separated on a 0.7% agarose gel, along with DNA Molecular Weight Marker II Digoxigenin. The DNA was then transferred to a nylon membrane according to the method described by Sambrook et al. (1989). Once the DNA was separated on a 0.7% agarose gel, the gel slab was placed in a denaturing solution for 45 minutes, and gently shaken, using a platform shaker. It was then washed in dH_2O for 10 minutes and placed in a neutralising solution for 30 minutes. Following this, the gel was then placed on pre-soaked chromatography paper in 20xSSC on a gel tray (Figure 2.3). Nylon membrane (Hybond-N, Amersham) and was then cut to the same size as the gel and placed on top of the agarose gel. Two sheets of chromatography paper were cut to size and soaked in 20xSSC, and then placed on top of the membrane. After removing any bubbles present between the gel, the nylon membrane and chromatography papers, many dry chromatography papers (two-three inches), were placed on top, with a weight placed above the filter paper to hold it in place and allow the DNA transfer onto the membrane by capillary action. Following the overnight transfer period, the DNA was fixed to the nylon membrane by exposing the membrane to UV light (UV Stratalinker 2400) for 1 minute. The membrane was then placed in Saran wrap, to dry, and then stored in the dark at RT until required.

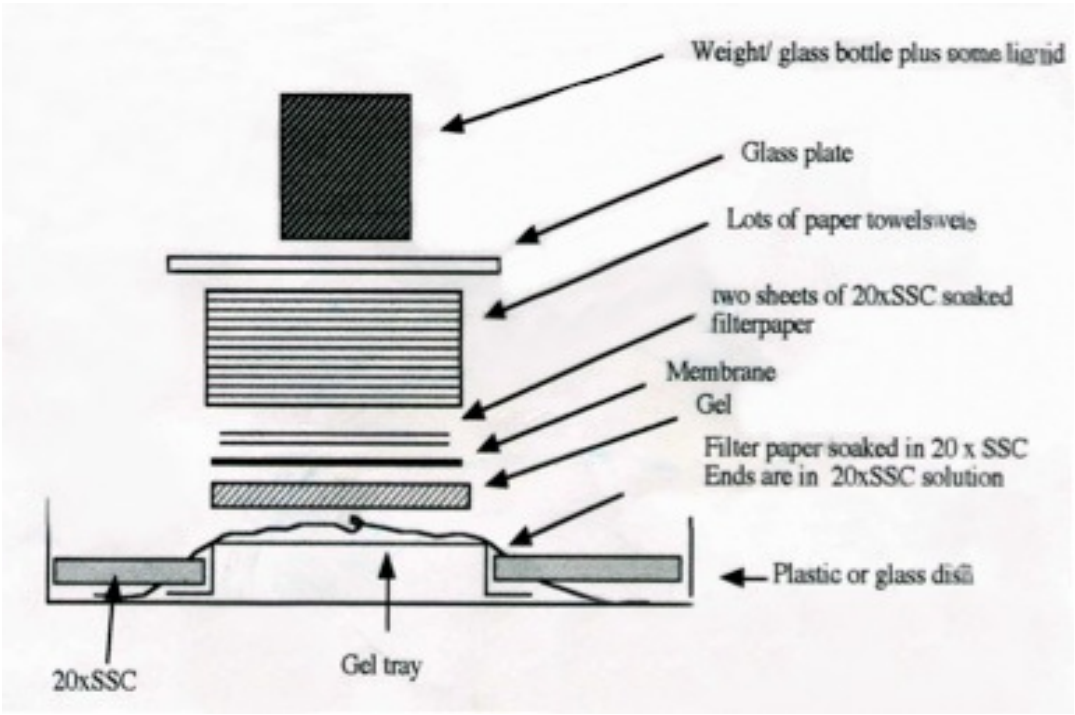


Figure 2.3 A diagram, highlighting the transfer of DNA to a nylon membrane by capillary action.

DNA hybridisation was performed in a hybridisation oven (Shaker S1 20H, Stuart Scientific). Firstly, the nylon membrane was placed in hybridisation solution for 1hr at 62 ^{o}C with slow shaking. The Dig-labelled probe was denatured; 10µl probe and 90µl sdH_2O (95 ^{o}C / 5 minutes). The denatured probe was then swiftly placed on ice, and then added to the hybridisation solution (indirectly onto the nylon membrane). For the next step, the nylon membrane was then incubated in the hybridisation solution with the probe, for 24 hrs at 62ºC, with gentle shaking. Following the hybridisation step, the membrane was washed as follows; twice for 5 minutes each at RT with a low stringency wash buffer (2xSSC+0.1% (W/V) SDS) slow shaking and then twice with high stringency washes (0.1xSSC+0.1% (W/V) SDS) at 62 ºC/15 minutes with shaking. Then the membrane was stored in a dark drawer until required for the detection steps.

Subsequent detection of DIG labelled DNA steps was conducted at RT as follows; the membrane was washed in DIG buffer 1 (5 minutes with slow shaking), and then submerged in Buffer 2 (100 ml) for 30 minutes with gentle shaking. Another brief wash with Buffer 1 was completed and followed by incubation in 30ml of buffer 1 and 6 µl antibody solution; Anti-DIG conjugated alkaline phosphatase (Roche) for 30 minutes with gentle shaking. Next, the membrane was washed twice in 100ml of Buffer 1 (10 minutes each with slow shaking), and was equilibrated by placing it in 100ml of Buffer 3

(2 minutes with slow shaking). Finally, the membrane was placed in a square Petri dish, with 90ml of development solution and left in the dark up to for 2- 3hrs, until the colour developed. Then, the membrane was washed with H_2O and stored in a dark drawer.

Denaturing Solution:

NaCl	43.83 g
NaOH	10 g
Distilled water	Up to 500ml

Neutralising Solution:

NaCl	43.83 g
EDTA (0.1M soln)	1ml
Tris/ HCl pH 7.2 (1M soln)	250ml
Distilled water	Up to 500ml

20x SSC:

Na_3Citrate	44.1 g
Distilled water	Up to 500ml
pH to 7 with 1N NaOH	

Hybridisation Solutions:

5x SSC	5ml 20x SSC stock solution
0.02% SDS	20µl 20% SDS stock solution
I% blocking reagent	2ml 10% Skimmed milk stock solution
Distilled water	Up to 20ml

Detection DIG labelled DNA Solutions:

10xBuffer 1

100 mM Tris/ HCl pH 7.5	121.1 g
150 mM NaCl	87.7 g
Distilled water	Up to 1000ml
pH to 7.5 with 1N NaOH	

10xBuffer 2:

100 mM Tris/ HCl pH 7.5	121.1 g
150 mM NaCl	87.7 g
I% blocking reagent	100ml 10% skimmed milk stock solution
Distilled water	Up to 1000ml
pH to 7.5 with 1N NaOH	

Buffer 3:

100 mM Tris/ HCl pH 9.5	12.11 g
100 mM NaCl	5.849 g
50 mM $MgCl_2.6H_2O$	10.17 g
Distilled water	Up to 1000ml
pH to 9.5 with 1N HCl	

Development Solution:

NBT Solution:	115µl
75 mg/ml nitroblue tetrazolium in 70% (v/v) dimethyl-formamide	
X-phosphate (BCIP) Solution:	90µl
50 mg/ml 5-bromo-4-cholor-3-indolyl phosphate (BCIP) in dimethyl formamide	
Stored @ -20 ^oC	

2.2.13 Preparation and transformation of chemically competent cells

2.2.13.1 Preparation of chemically competent *E. coli* cells

This was performed using a calcium chloride method of Sambrook et al. (1989), with some modification as follows; single colonies of *E. coli* strains (Section 2.1.1.3) were inoculated into 5ml of LB (+ appropriate antibiotic; 20 µg/ml St for λ*pir* and 50µg/ml Tet for XL1-*Blue*, Section 2.1.1.3) and aerobically incubated by shaking (overnight at 37 ^{o}C).

The fresh overnight *E. coli* strains (λ*pir* or XL1-*Blue*) were sub-cultured in 5ml of appropriate LB broth media. Following the period of incubation, 1ml of overnight culture was transferred into 100 ml of LB broth media and incubated at 37 ^{o}C for 2-3hours (OD_{600}~ 0.6, exponential phase). The flask was then placed on ice for 15 minutes, to prevent growth. The culture was then divided into four 50ml sterile centrifuge tubes and centrifuged at 4000 rpm for 10 mins at 4 ^{o}C. The pellet was re-suspended in 10ml of ice-cold sterile 75mM $CaCl_2$, and left on ice for 20 minutes, the cells were then centrifuged as before. The pellet was then re-suspended in 4ml of transformation buffer TFB2 (10 mM 3-(N-morpholino) propanesulfonic acid (MOPS) [pH 7.0], 75 mM $ClCl_2$, 10 mM RbCl and 15% Glycerol) and incubated on ice as before. The competent *E. coli* cells were then dispensed into 1.5ml microcentrifuge tubes (400µl per tube) and stored at -70 ^{o}C until needed for transformation.

2.2.13.2 Transformation of Competent Cells

Recombinant plasmids (cloned DNA) were transferred to chemically competent *E. coli* strains (λ*pir* or XL1-Blue,) using the heat shock method defined by Sambrook *et al.* (1989). Up to 10µl of plasmid DNA (10-50ng, Section 2.2.2) was mixed with 100µl thawed *E. coli* competent cells (from -70 ^{o}C freeze stock above) and 50µl TMC buffer (10 mM Tris/ HCl [pH 8.0], 10 mM $MgCl_2$ and 10 mM $CaCl_2$), in a 1.5ml microcentrifuge tube. The mixtures were then placed in a water bath at 42 ^{o}C for 60 seconds and then immediately placed on ice (5 minutes). 1ml of sterile LB broth was added to the mixture and the cells incubated aerobically at 37 ^{o}C for 30 minutes. Cells were harvested with

centrifugation (13000 r.p.m. for 5 mins at RT) and re-suspended in the residual media (~50µl). Following this, the cells were spread onto LB agar plates with appropriate antibiotic selection. Non-transformed competent cells were also plated out as a negative control. Plates were incubated overnight at 37 ^{o}C.

2.2.14 Bacterial mutagenesis (Tn5 mutagenesis)

2.2.14.1 Tn5 mutagenesis

In this study, a transposon mutagenesis technique was used to isolate potential non-antimicrobial mutant strains (PM) (Larsen *et a*l., (2002). *S. plymuthica* (P), was used as a recipient, in conjunction with the *E. coli* donor strain (BW20767 (carrying the Tn*5*-pRL27 ((Km^{R}-*ori*R6 K)) plasmid (Figure 2.4) (Larsen et al., 2002).

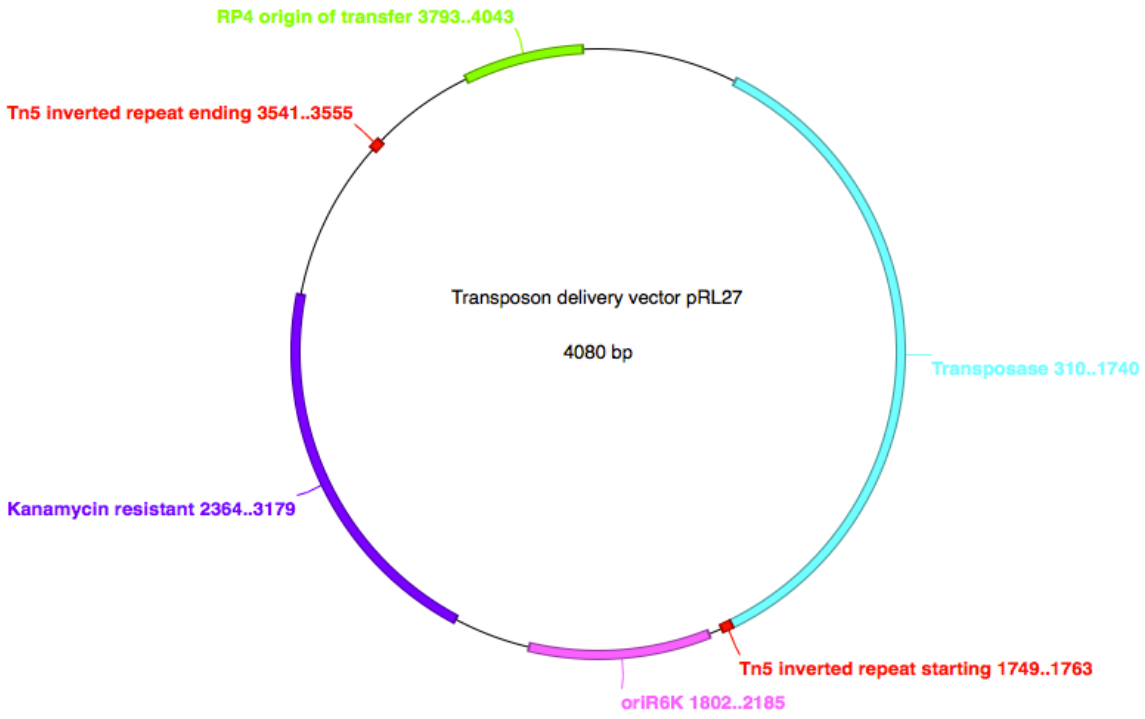


Figure 2.4 Graphic map (circular) of delivery vector (pRL27) plasmid.

An overnight culture of the recipient strain was grown in nutrient broth medium at 21 ^{o}C, while an overnight culture of the donor strain (*E. coli*) was grown in a selective broth medium (LB 50µg/ml Kan) at 37 ^{o}C. Both cultures were aerobically incubated by shaking (170 rpm). Following this, fresh cultures of both the recipient (P) and the *E. coli* donor, were prepared by inoculating 5ml of fresh broth media with 0.1ml of each overnight culture and incubating aerobically, at the appropriate temperature, until reaching an OD_{600} of ~ 0.8 to achieve the same cell density (end –log phase). 1ml of the donor strain was transferred to a microcentrifuge tube and the cells pelleted by centrifugation. Following this, the cells were washed three times in 1ml sterile dH_2O. After which, the recipient cells and donor strain were mixed at a ratio of 1:1 (100µl of recipient (P) and 100µl of *E. coli* donor), in a 1.5ml microcentrifuge tube. For the next stage, the cells were mixed by vortexing for 10-20 seconds (Conjugation step starts). A 20µl volume of the mixed strains was then spotted directly onto the surface of the nutrient agar plates and incubated at 37 ^{o}C for 24hrs. Following this period of incubation, the colonies were then scraped off using a plastic inoculating loop, and resuspended in 10ml of sterile maximum recovery diluent (MRD, Section 2.1.3) and stored at 4 ^{o}C until required for further investigation. Figure 2.5 highlights the procedures used in the conjugation experiments.

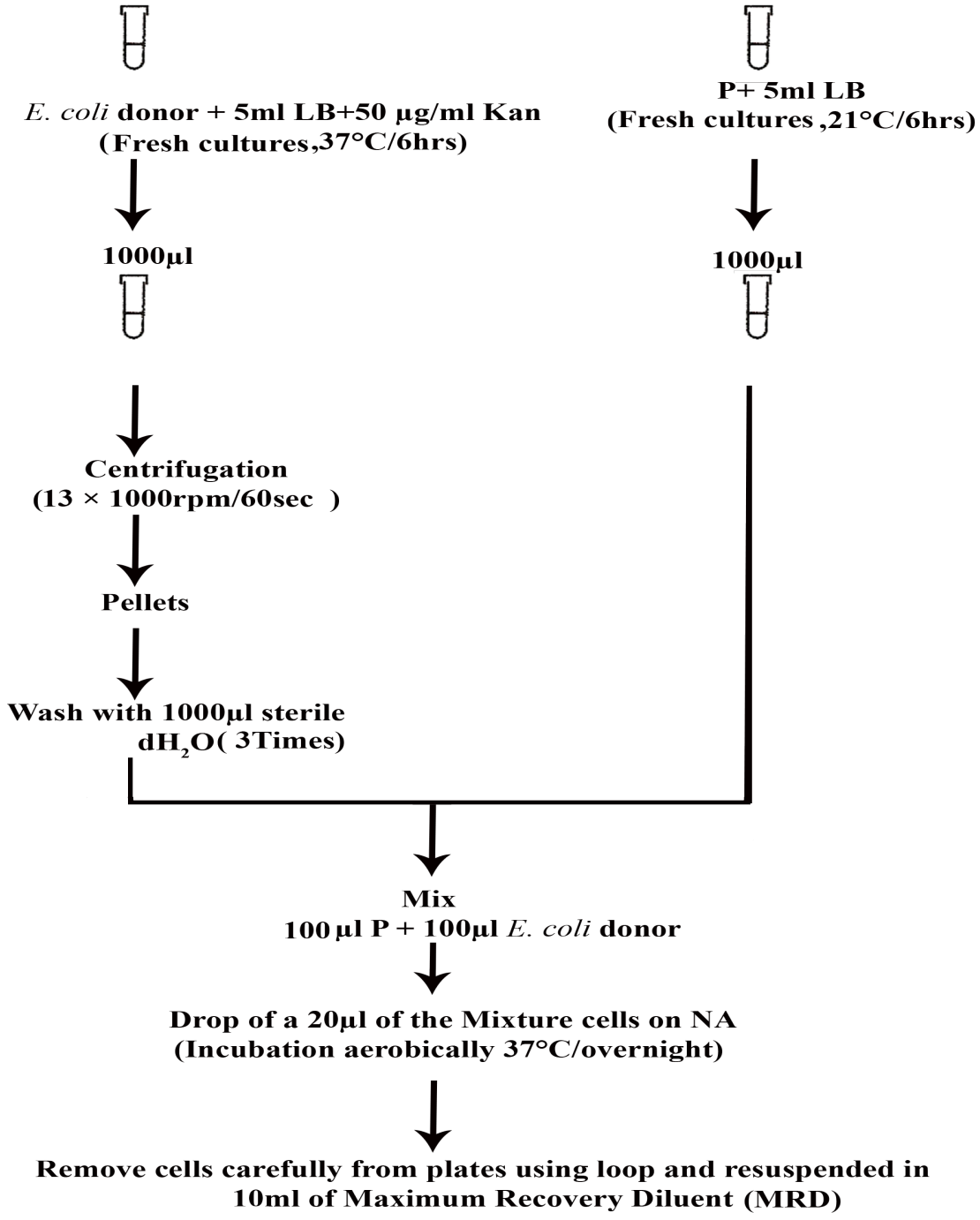


Figure 2.5 Random transposon mutagenesis protocol.

2.2.14.2 Screening for potential mutants

Nutrient agar plates containing two antibiotics (20µg/ml Ap, and 50µg/ml Kan) were used to screen for potential mutants after a mating experiment. Briefly, 100µl of each strain was plated out onto to the Nutrient agar, containing two antibiotics (20µg/ml Ap, and 50µg/ml Kan). The colonies growing on the selective plates were isolated using a sterile inoculating loop, and transferred to 96 well microtiter plates under microbiological conditions. Each well, contained 200µl of selective Nutrient broth medium (50µg/ml Kan and 20µg/ml Amp). The inoculated microtiter plates were then incubated under aerobic conditions, at 21 ^{o}C for 24hrs. Following the period of incubation, the kanamycin resistant trans-conjugants were then screened to identify those that failed to produce a clearing zone on a lawn of sensitive bacterial strain, using a spot-test (Section 2.1.5) with certain modifications. Square NA plates were used instead of standard Petri dishes, and were inoculated with 300µl of an overnight culture of the sensitive strain. A 96 pin sterile replicator was used to sample the microbial cells grown on the microtiter plates, onto these NA plates containing the sensitive strain. The plates were incubated aerobically at 21 ^{o}C for 24hrs. Potential mutants were then retested, to confirm the phenotype. A viable cell plate count was also performed, using the spread plate method (Section 2.1.8). Figure 2.6 shows the procedures used to screen for the potential mutant strains (PM) in this study.

10ml MRD+ Potential conjugated cells

↓

1ml into normal saline (9ml)
10^{-1}

↓

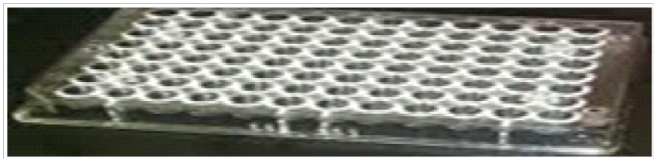
Further serial dilutions
$10^{-6} \rightarrow 10^{-2}$

↓

Plating the serial dilution2× 0.1ml (10^{-4}, 10^{-5}, 10^{-6})
1) Directly plating out 0.1ml onto NA
2)Directly plating out 0.1ml onto NA + 50 µg/ml Kan and 20 µg/ml AP
Incubation aerobically @ 21°C for 24hrs

↓

Obtain the non antimicrobial producer's colonies by a 96 well microtiter plates
Each well (0.3ml onto NA + 50 µg/ml Kan and 20 µg/ml AP)



Incubation aerobically @ 21°C for 24hrs with shaking

↓

96pin sterile replicator to screen for the mutants

↓

Square Nutrient ager plate with sensitive strain
Incubation aerobically @ 21°C for 24hrs

Figure 2.6 Screening for the potential mutant strains (PM).

2.2.14.3 Cloning and recovery of transposon insertion mutants

Figures 2.7 and 2.8 provide a diagram to summarise the process for cloning the transposon along with flanking genomic DNA. Genomic DNA of potential mutants was extracted (Section 2.2.1), and then digested (Section 2.2.4) (Larsen *et al*., 2002). Subsequently, the digested chromosomal DNA was ligated (Section 2.2.5) and introduced into chemically competent *E. coli* strains (Section 2.2.13). The transformation mixes were then plated onto selective agar LB plates (+ Kan [50µg/ml]) and Str [30µg/ml]) and incubated overnight at 37 ^{o}C. The Graphic map of Tn5 Transposon is shown Figure 2.9 and the digesting map with unique enzyme is in Figure 2.10.

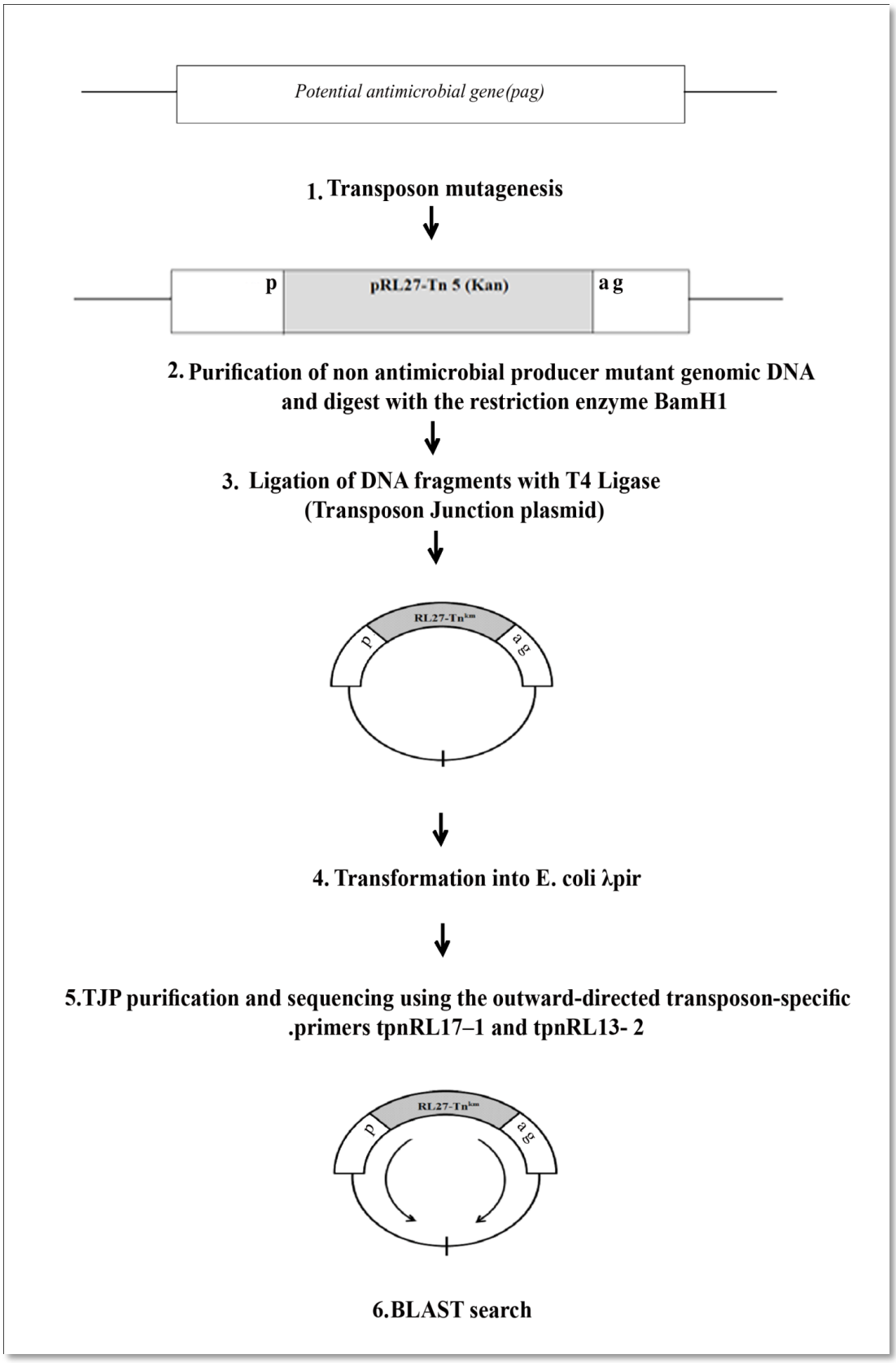


Figure 2.7 Diagram summarising the cloning, recovery and sequencing of the transposon junction plasmids (TJP).

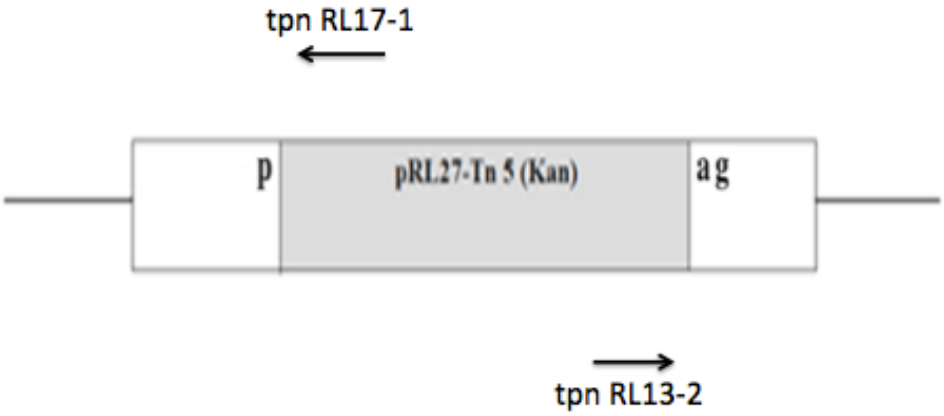


Figure 2.8 Diagram showing of primer sites (tpn RL17-1 and tpn RL13-2) to identify *pag*.

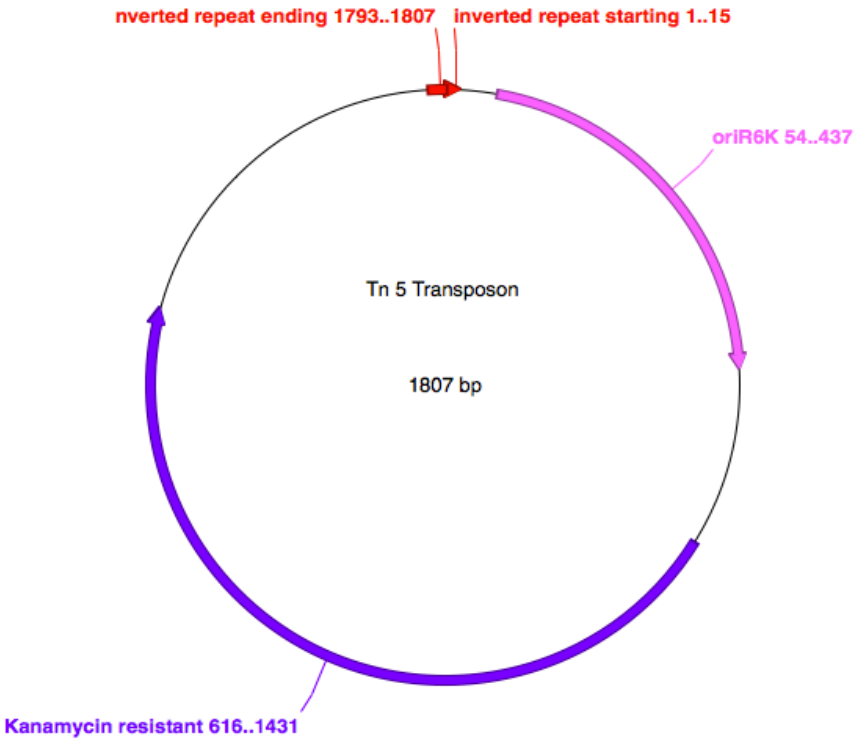


Figure 2.9 Graphic map (circular) of transposon.

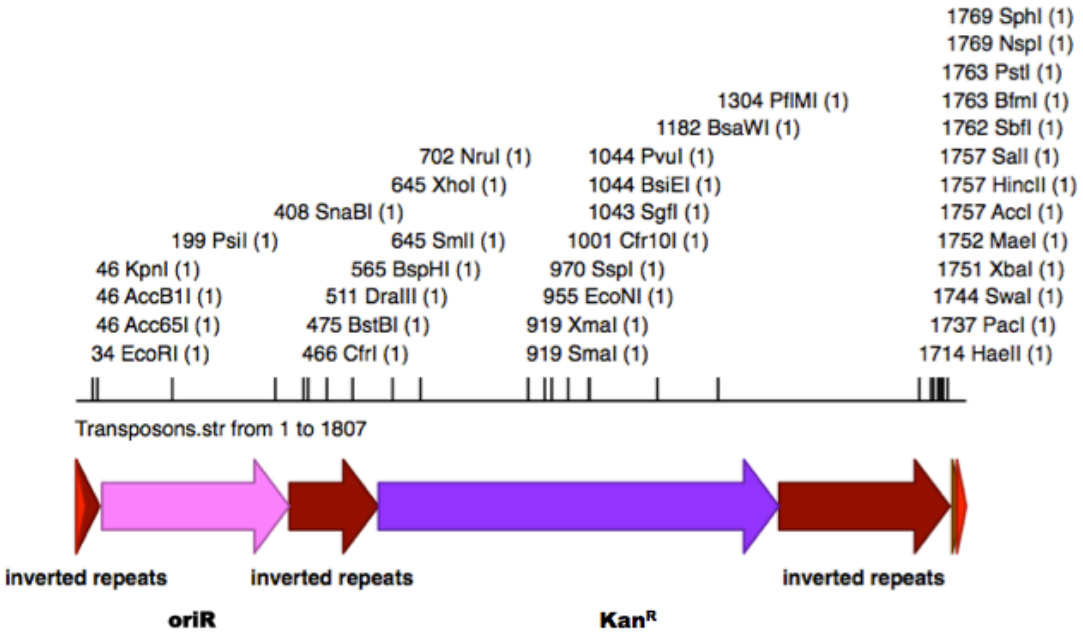


Figure 2.10 Graphic map (linear) of Transposons digesting map with unique enzyme.

2.3 Protein methods

2.3.1 Ultra filtration

The liquid samples (spin culture medium), was fractionated using a centrifugal concentrator technique; Vivaspin Ultra filtration spin columns (15ml, Biotech, Germany), with different molecular weight cut-off membranes (100,000; 50,000; 30,000; 10,000 and 5000 Da) according to the manufacturer's instructions.

2.3.2 Concentration of spent culture media

The spent culture medium (SM) was concentrated using a centrifugal vacuum concentrator (Savant AES 2010 Speed Vac® System). Briefly, 20ml of SM was distributed by pipette, into sterile 1.5ml microcentrifuge tubes. All the tubes were then placed into the vacuum concentrator and run for 3 hrs at RT. The dried materials in each

tube were then re-suspended in 50µl of sterile distilled water and labelled concentrated spent media (CSM) samples, and stored in the refrigerator until used.

2.3.3 Size exclusion chromatography (SEC)

A fast Protein Liquid Chromatography (FPLC) system was used in this study by employing a Superose®-12 column (GE Healthcare Life Sciences). The column was initially calibrated using the following molecular weight (MW) markers;

Compound	**MW/ Da**
Blue Dextran	2000000
Bovine serum albumin (BSA)	67000
Cytochrome C	12700
Vitamin B12	135537

Subsequently, 1ml of test sample such as concentrated spent media (CSM) of test strains resuspended in the following buffer; 50mM Tris (pH7.5), 150 mM NaCl and 1mM EDTA was injected and the Colum run at a flow rate of 0.8ml/min. The fractions were monitored with UV light, at 280nm, and collected in 1.5ml microcentrifuge tubes.

2.3.4 Anion exchange chromatography (AEC)

This was performed using the same methods, involved in size exclusion chromatography with modifications as follows; Mono Q column (GE Healthcare Life Sciences) (negatively charged) instead of a Superose®-12 column, and different buffers; Low salt (50mM Na acetate [pH 5] +100mM NaCl and high salt (50mM Na acetate [pH 5] +1M NaCl.

2.3.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.5.1 Sample preparation for SDS-PAGE

1ml overnight cultures were spun in a microfuge tube at 13,000 rpm for 1 minute. Then, the supernatant was discarded and pellets re suspended in 120µl 2X SDS PAGE loading buffer (sample buffer). For the next stage, protein denaturation was completed, by placing the sample on a heating block, at 95 ^{o}C for 5 minutes, with a further centrifugation applied, to pellet the cell debris. For the analysis of the cell free spent media, (SM), a 100µl of 2X SDS PAGE loading buffer was added to 100µl of test samples, followed by heating to denature the proteins (95 ^{o}C/5 minutes) in a heating block. Further centrifugation was carried out to remove any precipitate.

2.3.5.2 SDS PAGE

The SDS equipment was cleaned with water, then 95% ethanol. Next, a gel sandwich was assembled and placed in an alignment slot, with an alignment card, between glass plates. Following this, two 10% mini-gels were prepared. The resolving gel mixture was poured into the space between the glass plates (about 75% of the space) and covered with 50µl of water-saturated butanol (5ml butanol + 5ml H_2O). When the gel was set (about 20 minutes), the butanol was discarded and a 10-well comb inserted. The stacking gel was gently added using a pipette and left to polymerise for 10 minutes. Then, the comb was removed from the gel and placed in the SDS-PAGE chamber, and run in 1X running buffer after loading 15µl of SDS-PAGE prepared samples, (Section2.6.4.1) and 10µl of a molecular weight marker (26,600-1,060Da) solution (Sigma).

2.3.5.3 Staining of SDS page gels

Coomassie brilliant blue (CBB) was used to stain the SDS gel according to the method of Garfin, 1990. The gels were then removed from the SDS-PAGE chamber and placed in a plastic container containing staining solution, and left to stain for 2hrs by gently shaking them. Next, the gels were removed from the stain solution and placed on a plastic

container for destaining, using a destaining solution and left overnight at RT, after slowly shaking. The gel was then placed on clear background to detect blue-stained bands (proteins) and photographs were taken.

Resolving gel 20ml(10%):

4xResolving Buffer	5ml
40% Acrylamide/Bis'*	5ml
H_2O	9.28ml
APS (10%)	100µl
TEMED	20µl

*Sigma 40% Acrylamide/Bisacrylamide mix (Cat. No. A7802-100ML, Store @ 4 ^oC)

Stacking gel 10ml (3%):

4 x Stacking buffer	2.5ml
40% Acrylamide/Bis	0.75ml
H_2O	6.37ml
APS (10%)	50µl
TEMED	20µl

4x Resolving gel buffer:

Tris base	18.17g
10% SDS	1ml
H_2O	To 100ml

PH to 8.8 with HCl

4x Stacking gel buffer:

Tris base	6.06g
10% SDS	1ml
H_2O	To 100ml

pH to 6.8 with HCl

10x Running Buffer:

Tris base	30.28g
Glycine	144.25g
SDS	10g
H_2O	To 1 L (pH will be ~8.6)

Dilute it to 1X for running use

Sample buffer (2X LSB OR SDS loading buffer):

Tris (HCl) 0.5M pH6.8	6.25ml
SDS	2 g
Glycerol	9ml
2-mercaptoethanol	5ml
Bromophenol blue (0.1% soln')	10ml
H_2O	To 50ml

Stain:

Coomassie blue (R250)	0.5 g
Methanol	250ml
Glacial Acetic acid	50ml
H_2O	200ml

Destain:

Methanol	100ml
Glacial Acetic acid	100ml

2.4 Quorum sensing assay

The production of N-acylhomoserine lactones (AHLs) was evaluated using a cross-streaking bioassay as described by Swift et al. (1997). Briefly, fresh culture bacteria were cross-streaked against a biosensor strain *Chromobacterium violaceum* (CV0blu {Section 2.1.1.3.}) onto an NA plate using a sterile toothpick. The assay was conducted in triplicate and the plates incubated at (21 ^{o}C and 30 ^{o}C for between 1 and 2 days). A positive result was recorded by the production of a purple pigment in the test bacterial strain. *Chromobacterium violaceum* (CV031 {Section 2.1.1.3.}) was used as a positive control.

RESULTS

3. Identification of the marine bacterium 'P'

3.1 Introduction

At the present time, the increasing prevalence of antibiotic resistant "super-bugs" and risks associated with food safety are global issues. Of particular concern is the risk of food borne illnesses, which are in part due to the transportation of high water content foods that are easily spoiled as result of undesired microbial growth (food spoilage) (http://www.cdc.gov). Consequently, excessive use of antimicrobial agents has become an additional global issue, because many bacteria have developed resistance or have become increasingly resistant to commercially available antibiotics. Thus, there is a need for a continuous search to identify new and effective antimicrobial substances (Dessen *et al*., 2001; Alanis, 2005). Traditionally most antimicrobials were isolated from organisms in the terrestrial environment, with little attention given to marine based organisms, until relatively recent times. Marine based micro-organisms still represent a relatively untapped source of new antimicrobials (Stach, 2010). In view of this, seaweed was screened for associated marine microorganisms with the ability to produce antimicrobial compounds. An organism producing antibacterial metabolite(s) was found to be strongly active against several microorganisms, particularly the microorganisms associated with food borne spoilage, food borne pathogens and multi-resistant human pathogenic bacteria, such as VRE and MRSA.

Morphological, phenotyping and ribotyping experiments were carried out to identify the isolated marine bacterium (P), using classic microbiology and molecular microbiological techniques, as follows:

3.2 Results

3.2.1 Screening for Antimicrobial Compound Production

During a screening of seaweed associated marine micro-organisms, for their ability to produce antimicrobial compounds, an organism was isolated, as producing antimicrobial metabolite(s) and was termed P. This marine isolated micro-organism was screened for its ability to produce antimicrobial compounds and kill or prevent the growth of a variety of different bacterial and fungal strains. Antimicrobial activity was performed in this study using two methods; the disc diffusion test (Mearns-Spragg *et al*,. 1998) and also the spot test (Material and Methods, Section 2.1.5) ,against various of test micro-organisms (Table 2.1). The results from these experiments demonstrated that this marine bacterium strongly inhibited the growth of the test micro-organisms particularly, multi-drug resistant *Staphylococcus aureus* (MRSA)*, Clostridium difficile* strain 630, Vancomycin resistant *enterococci* VRE, *Bacillus cereus var mycoides, Listeria monocytogenes* scottA, *Staphylococcus epidermidis* and the food spoilage fungus, *Penicillium expansum.* The data is shown in Table 3.1 with selected images of the zones of growth inhibition shown in Figure 3.1. Based upon these results, it was decided to investigate this antimicrobial producer strain (P) in greater depth to increase our knowledge about this strain.

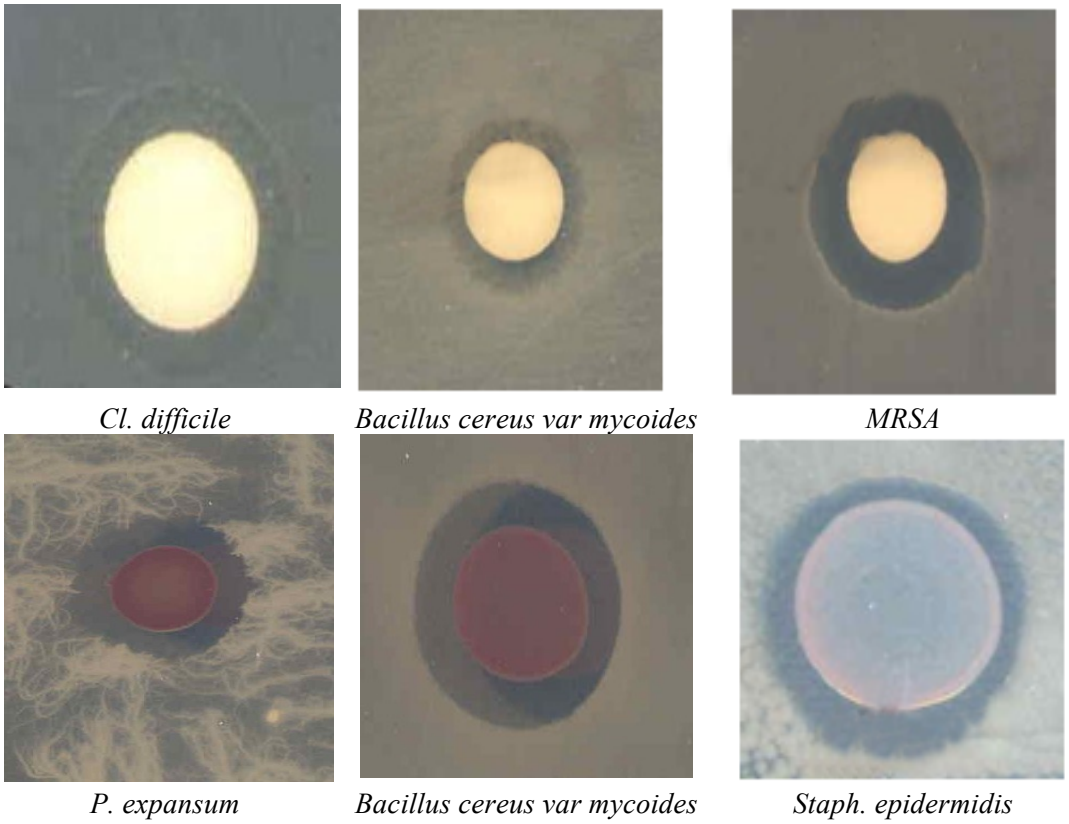


Figure 3.1 Disc test (top panels, 100 µl CSM was added to a sterile paper disc in the center of an NA plate spread with a lawn of *Cl. Difficile*, *Bacillus cereus var mycoides* and MRSA (as the sensitive strain)) and a spot test (bottom, 10µl volume of a fresh overnight culture of the test microorganism (P) was spotted directly onto the surface of an NA plate spread with a lawn of *P. expansum, Bacillus cereus var mycoides* and *Staph. epidermidis* (as the sensitive strain)), showing the antimicrobial effect of marine isolate P.

Table 3.1 Summary of antimicrobial activities of the marine isolate P, filter-sterilised spent culture medium (SM), and concentrated spent media (CSM), against wide varieties of indicator micro-organisms. For the tests/various spent media were prepared, see Material and Methods, Section 2.1.5 and Results, chapter 5, Section 5.2.1.

Micro-organism	**Antimicrobial activity**			**Growth conditions**
	Spot test (10µl) SM	**Spot test (20µl) SM**	**Disc test (100µl) CSM**	
L. monocytogenes scottA	+++	+++	+++	30 °C /24h - NA - aerobic
L. monocytogenes NCTC7973	++	+++	+++	30 °C /24 h - NA - aerobic
B. cereus var mycoides (F)	+++	++	++	21 °C /24 h - NA - aerobic
S. epidermidis	+++	+++	+++	30 °C /24 h - NA - aerobic
MRSA	+++	+++	+++	30 °C /24 - NA - aerobically
VRE	++	++	++	30 °C /24 h - NA - aerobic
Clostridium difficile	+++	+++	+++	25 °C /48 h - NA - anaerobic
Penicillium expansum	+++	++	++	25 °C /4 days – NA and YPD-aerobic
Escherichia coli	-	-	-	35 °C /24 h- NA - aerobic
E. coli (D28)	-	-	-	35 °C /24 h- NA - aerobic
E. coli BW20767	++	++	++	
Pseudomonas aeruginosa	-	-	-	30 °C /24 h- NA – aerobic

Table 3.1 continued

Candida albicans (NCTC3153)	-	-	-	30 °C /24 h- NA and YPD-aerobic
Candida albicans NCTC SC5314)	-	-	-	30 °C /24 h- NA and YPD-aerobic
Pichia angusta	-	-	-	30 °C /24 h – NA and YPD-aerobic

(+++)=(3mm) Very strong antimicrobial activity, (++)=(2mm) strong antimicrobial activity, partial antimicrobial activity (+)=(1mm) and (-)=(0mm) no antimicrobial activity.

3.2.2 Bacterial identification

Based on the findings presented in the previous Section (3.2.1), it was decided to identify marine isolate P. The use of phenotypic profiling should provide useful information about the group and species that the strain (P) belongs to. Potentially this will help identify the inhibitory molecule(s) produced by this strain. The tests performed to identify P are detailed below:

3.2.2.1 Colony morphology

On agar plates the bacterium formed circular, convex, brick red pigmented and smooth surface colonies (Figure 3.2). Gram staining revealed that its cells were short rod shaped, gram-negative, and non-spore forming.

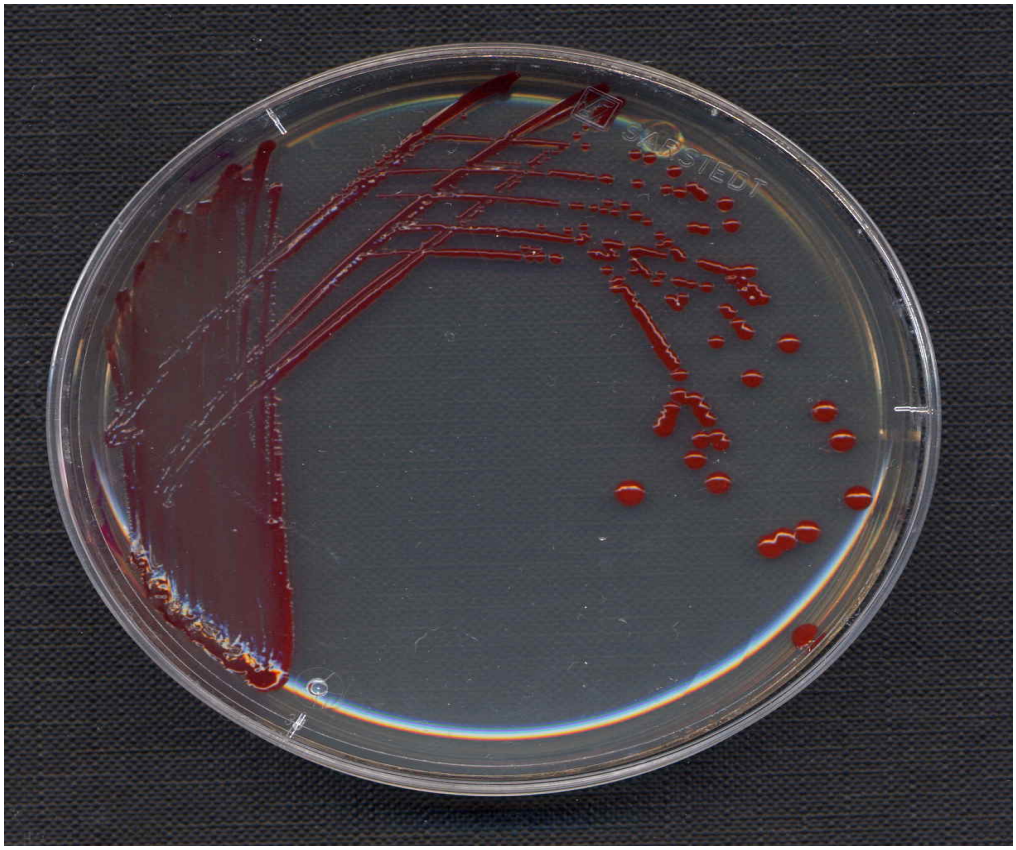


Figure 3.2 Pure culture of marine isolate P (antimicrobial producer) on a NA plate, grown at 21 ^{o}C for 24hrs.

3.2.2.2 Salt Tolerance

To obtain more detailed information about the properties of the antimicrobial producer strain (P), a salt tolerance assay was carried out. P was isolated from the marine environment and as a result I decided to determine how salt tolerant it was? 10 ml of NB was inoculated with a single colony of strain P, and aerobically incubated by shaking (overnight at 21 ^{o}C). Following the period of incubation, 100µl the overnight culture was transferred into 10ml of nutrient broth, supplemented with increasing amounts of NaCl (5g/l, 10g/l, 15g/l, 20g/l, 25g/l and 30g/l) and incubated at 21 ^{o}C for 8hrs (OD600nm= 0.830, late exponential phase). The colony forming units (CFU) were counted per ml for each concentration, following the incubation period (21 ^{o}C for 18hrs) (Material and Methods, chapter 2, Section 2.1.8). The results were recorded as survival curve (% CFU/ml), against NaCl concentrations. The result showed that marine isolate P is a salt-tolerant bacterium (Figure 3.3).

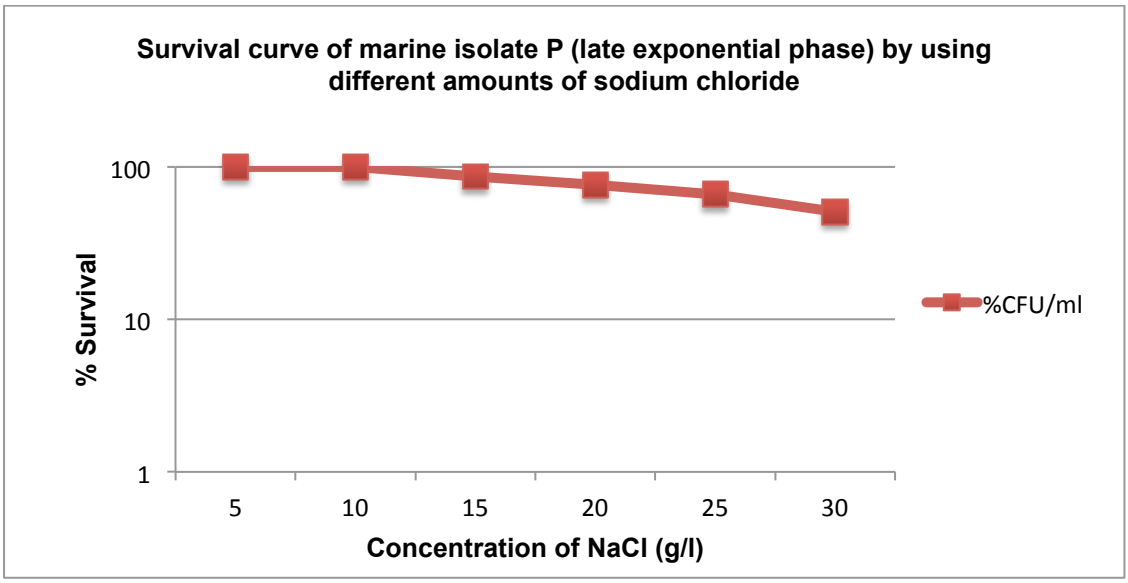


Figure 3.3 Cell survival of marine isolate P cells on exposure to increasing concentrations of sodium chloride (g/l). Bacterial cultures were grown aerobically in nutrient broth, supplemented with increasing amounts of NaCl (5g/l, 10g/l, 15g/l, 20g/l, 25g/l and 30g/l) and incubated at 21 ^{o}C for 8hrs (OD600nm= 0.830, late exponential phase). Total viable cell count was then carried out on NA plates and these were incubated overnight at 21 ^{o}C. The result represents a survival curve (% CFU/ml) against increasing concentrations of NaCl. N= 2 times.

3.2.2.3 Production of extracellular enzymes

Many micro-organisms, including fungi, actinomycetes, as well as bacterial species, have the ability to secrete cell-wall hydrolase enzymes such as chitinase and proteases (Frankowski *et al*., 2001). As a result this bacterium (P) may produce extracellular enzymes which could possibly explain the antimicrobial activity. Therefore, production of extracellular enzymes was examined. Strain (P) was investigated for its ability, to produce several enzymes, such as chitinase, proteinase, lipase, casinase, gelatinase and DNase. An overnight fresh culture of the bacterium P was spotted onto fresh agar plates; Gelatin agar, Casine agar, DNA agar, Colidal-chitin agar, Tributyrin agar and blood agar, and incubated for up to 24 hrs/21 ^{o}C. The results shown in Figure 3.4 show that the marine bacterium P was chitinase positive, proteinase positive, lipase positive, gelatinase positive, DNase positive and haemolysis positive.

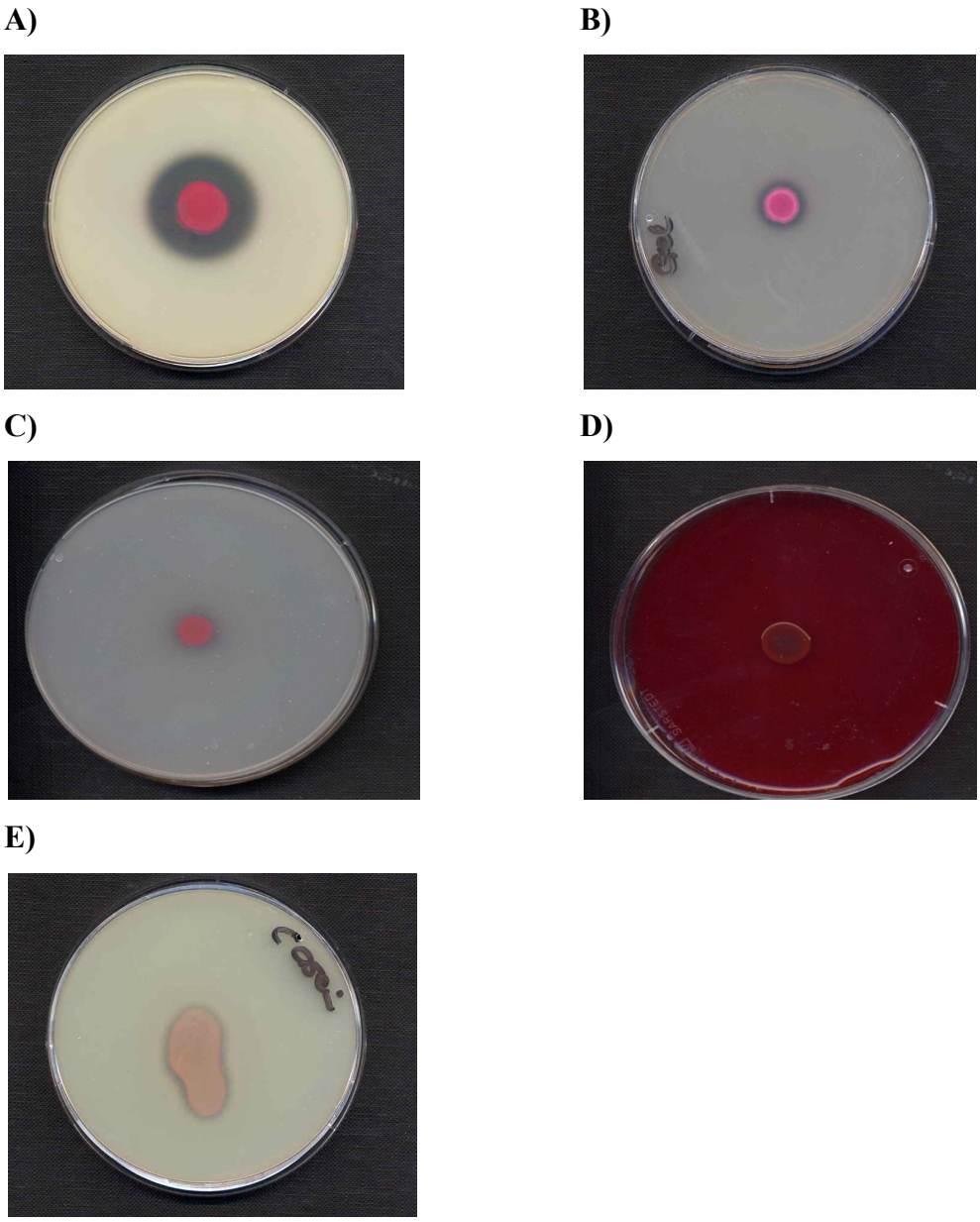


Figure 3.4 Enzymatic producing assays. 10µl of a fresh broth culture of marine isolate P was placed on agar plates to assay for the presence of key enzymes; A (DNase activity, DNA agar), B (gelatinase activity, Gelatin agar), C (chitinase activity, colloidal chitin agar), D (haemolytic activity, blood agar) and E (casinase activity, casein agar), grown at 21 ^{o}C for 24hrs.

3.2.2.4 The effect of growth medium on the production of antimicrobial activity

It has been reported that the chemical composition of growth media can significantly affect the antimicrobial activity of some bacteria (Martin and Demain, 1980; Farmer, 1985; Spížek and Tichý, 1995; Marwick *et al*., 1999). Therefore, it was decided to study the effects of different growth media (NA, LB, TSA and BHI) on the antimicrobial activity of the marine isolate P. The experiments were performed using a spot test as described in Section 2.1.5 with *B. cereus var mycoides* as the sensitive microorganism, and incubated for 18- 24 hrs/21 ^{o}C. It was observed that the inhibition zone around the culture drop was much wider on the nutrient agar plates when compared with the other media tested (LB and TSA and BHI). Thus, NA plates are a good test plate for demonstrating the zone of inhibition against micro-organisms, whereas the other test plates negatively affected the production of the active compound(s). On the other hand, the red pigmentation was more prominent on the BHI agar plates, compared with NA, LB and TSA media, with LB and TSA plates resulting in an intermediate level of red pigment (Figure 3.5).

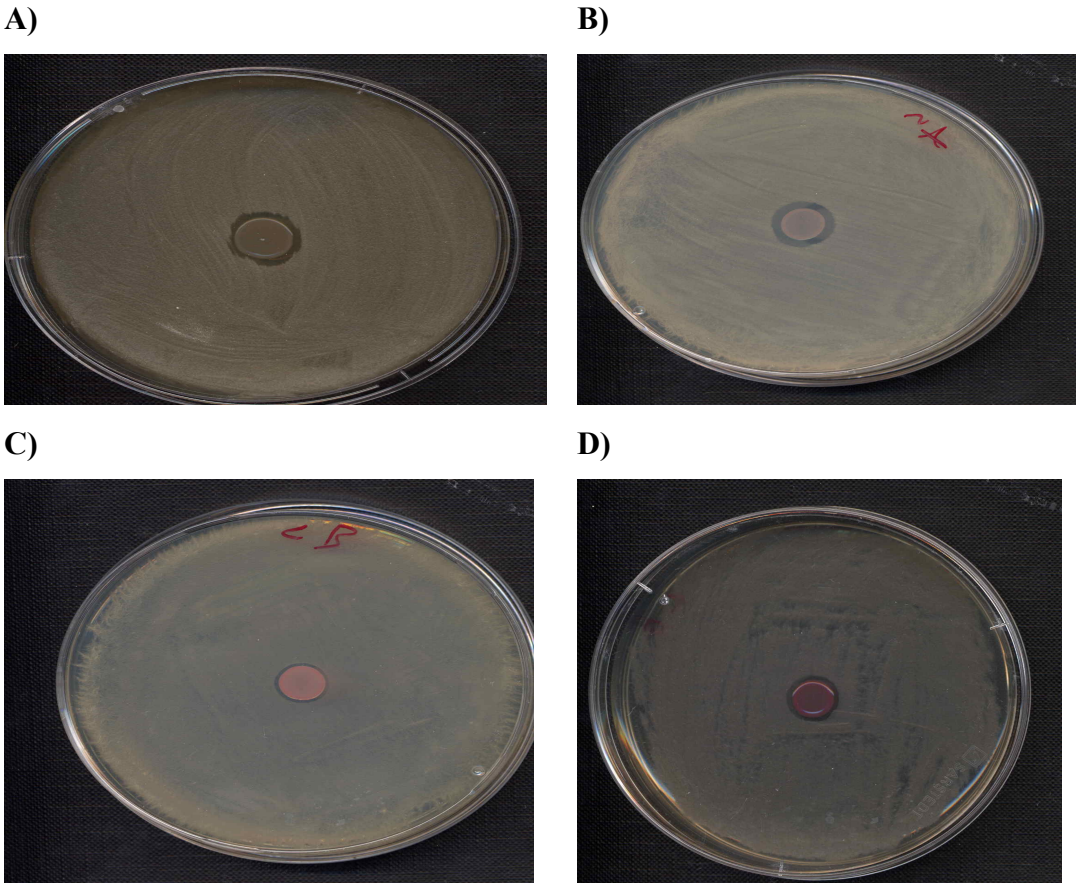


Figure 3.5 Effect of growth media on antimicrobial activity; 10µl of a fresh broth culture of marine isolate P was spotted onto different growth media seeded with a lawn of *B. cereus var mycoides*; **A** (LB), **B** (NA), **C** (TSA) and **D** (BHI agar).

3.2.2.5 The effect of growth temperature on the production of antimicrobial activity

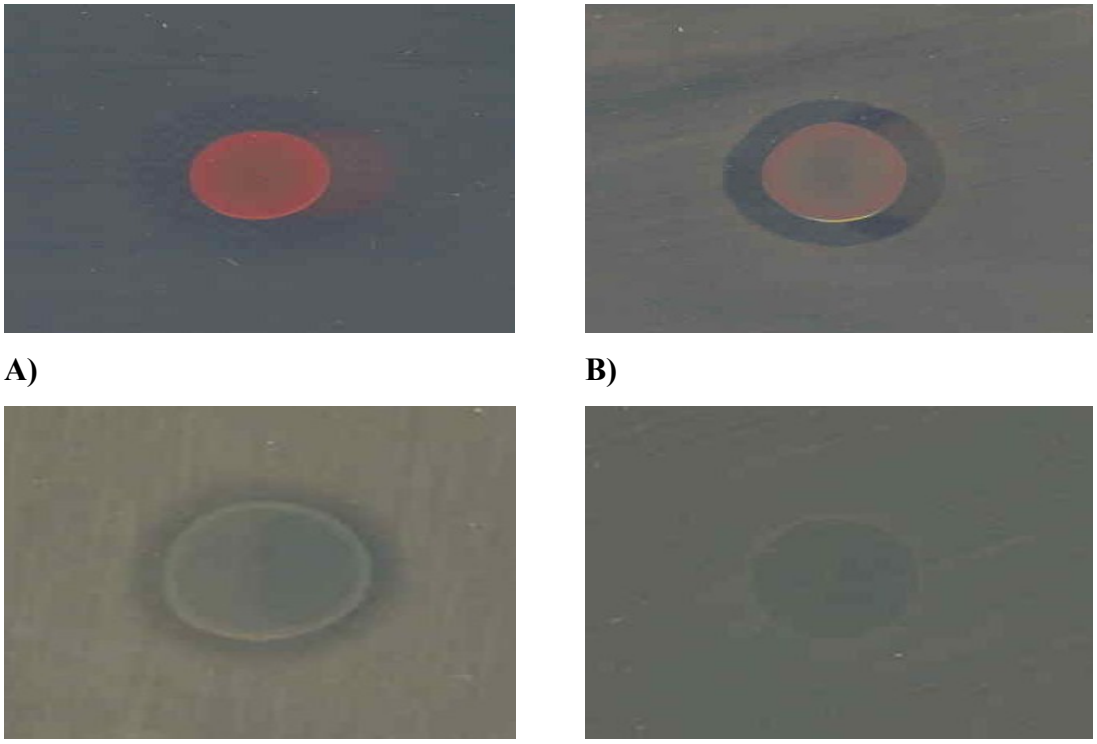
Exposure to external factors such as temperature can affect the functions of internal pathways, by controlling the expression of genes involved in the production of antimicrobial compounds (Moons et al., 2006). Therefore, the effect of various growth temperatures (9, 21, 30, 37 and 50^{o}C) on the growth, antimicrobial activity and red pigment production was tested in reference to the marine isolate P. The assay was performed using NA and NB media. Briefly, 10 ml of NB was inoculated with a single colony of strain P, and aerobically incubated at different temperatures (9, 21, 30, 37 and 50^{o}C) for up to 24hrs. Optical density (OD at 600_{nm}) was measured at 0h and 24hrs, respectively. Spot test NA plates were also prepared and incubated aerobically, at different temperatures (9, 21, 30, 37 and 50 ^{o}C) for up to 24 hrs and antimicrobial production and red pigmentation were recorded (Material and Methods, Section 2.1.5).

The results demonstrated that 30 ^{o}C was a better temperature for the growth of bacterium P, whereas 9 and 21 ^{o}C resulted in greater antimicrobial production (as demonstrated by larger zones of clearing). The results also highlighted that the level of red pigmentation reduced as the incubation temperature was increased (Table 3.2 and Figure 3.6).

Table 3.2 The effect of various temperatures on the growth, antimicrobial activity and red pigment production of marine bacterium P.

Growth temperature	**Antimicrobial activity**	**Red pigment production**	**OD 600nm**	
			0h	24h
9 ^{o}C	+++	+++	0.032	0.073
21^{o}C	+++	+++	0.092	0.620
30 ^{o}C	++	++	0.084	0.688
37 ^{o}C	-	+	0.075	0.326
50 ^{o}C	-	-	0.105	0.097

(+++) Very strong antimicrobial activity / red pigmentation , (++) strong antimicrobial activity / red pigmentation, (+) partial antimicrobial activity / red pigmentation and (-) no antimicrobial activity /no red pigmentation.



A) **B)**

C) **D)**

Figure 3.6 Effect of growth temperature on antimicrobial production and red pigment production; 10µl of a fresh overnight broth culture of marine isolate P was spotted onto NA agar plates, previously seeded with a lawn of *B. cereus var mycoides,* plates were incubated at the following temperatures; A (9 ^{o}C), B (21 ^{o}C), C (30 ^{o}C), and (37 ^{o}C) for 24hrs.

3.2.2.6 Antibiotic sensitivity tests

The spectrum of sensitivity towards a variety of antibiotics was also determined as this would help with further molecular microbiological techniques. Antibiotic sensitivity testing was performed using disc diffusion testing on NA plates, and the plates were incubated aerobically at 21 ^{o}C for 18-24hrs. The marine isolate demonstrated sensitivity to the following antibiotics; colistin sulphate, gentamicin, streptomycin, sulphatrid, tetracycline, cotrimoxazole, chloamphenicol, but was resistant to ampicillin, cephalothin and penicillin (Figure 3.7). In separate experiments I showed that P was also sensitive to kanamycin (data not shown).

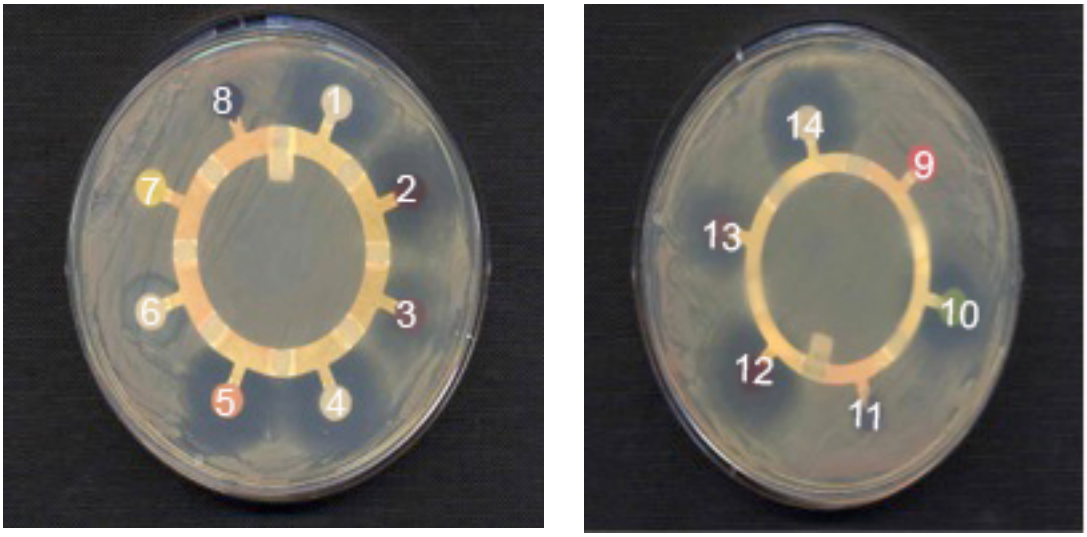


Figure 3.7 Antibiotic sensitivity disc tests for the marine isolate P; Strain P was sensitive to: Colistinsulphate (1), Gentamicin (2, 12), Streptomycin (3,13), Sulphatrid (4), Tetracycline (5), Cotrimoxazole (10), chloramphenicol (14); but was resistant to Ampicillin (6), Cephalothin (7) and penicillin (8,11). Growth condition, 21 ^{o}C /24 -NA.

3.2.3 Ribotyping

In order to study the gene(s) responsible for the production of antimicrobial compounds and to identify the inhibitory molecules, it was important to identify bacterial strain P. This was achieved by ribotyping, at the same time the identity of the sensitive strain (F) was also determined (Figure 2.1, Materials and Methods). Ribotyping analysis was done by sequencing the 16S rRNA encoding gene, according to Charrier *et al*., (2006), using specific primers (27F and 1492R, Table 2.7). These primers were initially used in a PCR reaction using genomic DNA from P and F, as templates, to amplify the 16S rRNA gene. The PCR reaction was carried out as described in Section 2.2.6. The PCR products were then cleaned using a Purification Kit and prepared for sequencing as described in Section 2.2.13 and sent to the University of Edinburgh sequencing facility (Genepool). The resulting sequences were used to search the nucleotide sequence databases with the BLAST program (www.blast.ncbi.nlm.gov/blast.cgi). The results showed that the marine isolate P has a high sequence identity (99%) to *Serratia plymuthica*, whereas the sensitive strain F showed a high sequence identity (99%) to *Bacillus cereus var mycoides* (Table 3.3 and Figure 3.8).

Table 3.3 Summary of the ribotyping results for the marine isolates P, and F.

Sequenced organism	**Blast match**	**% of nucleotide sequence identity**
P	*Serratia plymuthica*	99 (777/781 bp)
F	*Bacillus cereus var mycoides*	99 (734/738 bp)

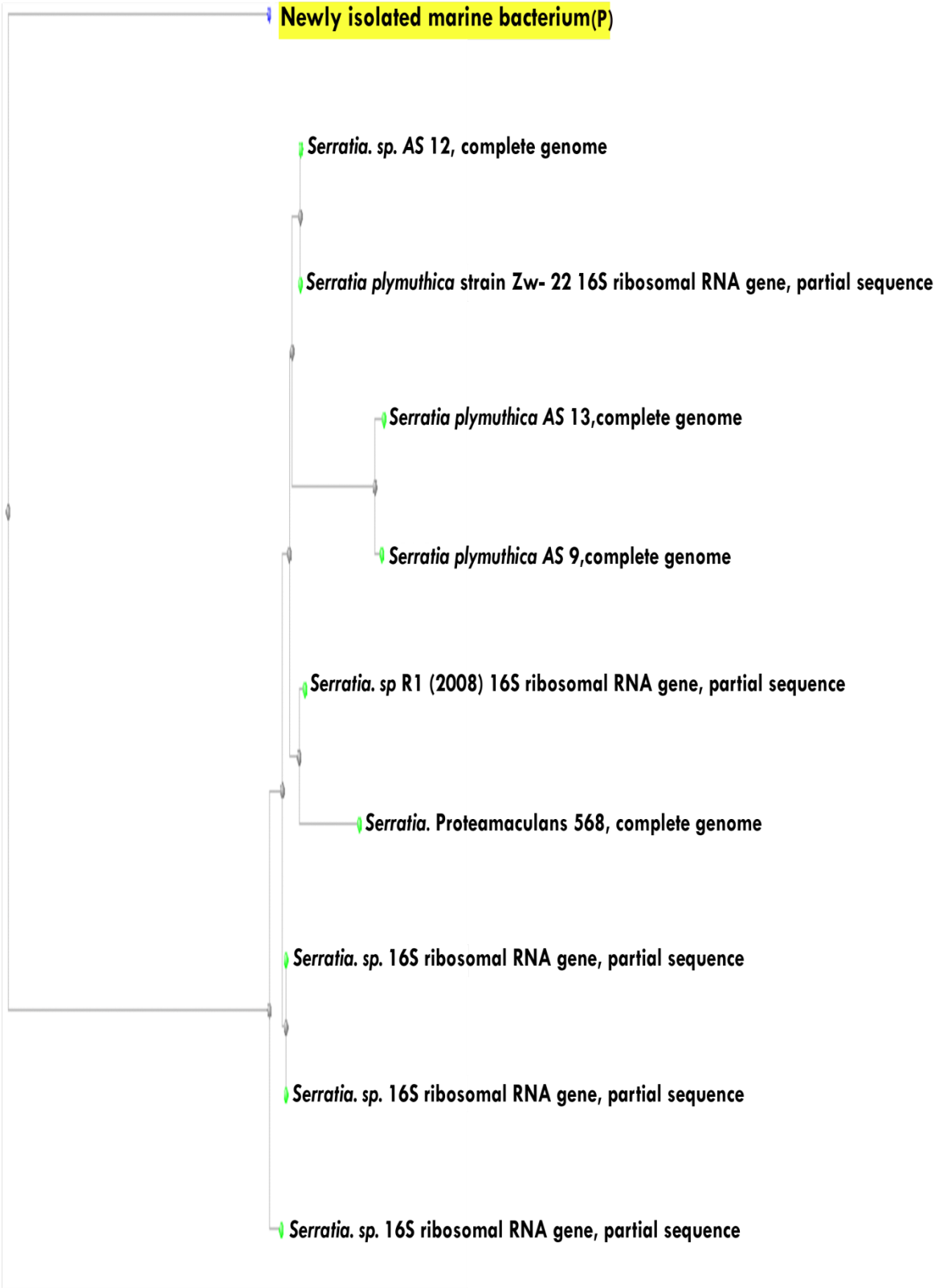


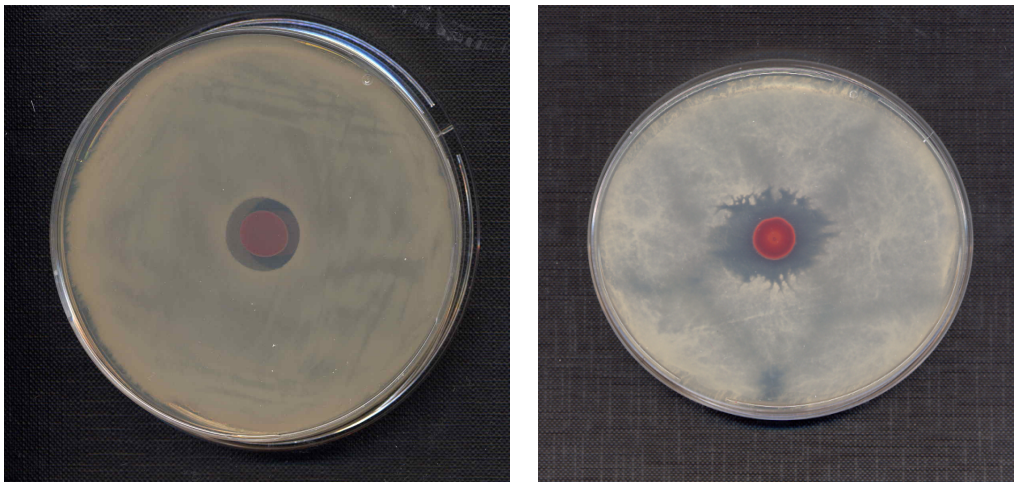
Figure 3.8 Phylogenetic tree based on the 16S rRNA gene sequences, highlighting that the marine antimicrobial producer is a variant of *Serratia plymuthica*.

In general, *Serratia plymuthica* species have received limited attention with respect to their antimicrobial producing properties, compared to *S. marcescens* (Stock *et al*., 2003;Harris *et al*., 2004). Studies performed on *S. plymuthica* strains have focused on the area of its potential pathogenicity in humans and animals (Nieto *et al*., 1990). However, a number of reports suggest that *S. plymuthica* have potential in the biocontrol of fungal pathogens (Compant *et al*., 2005; Meziane *et al*., 2006; Vleesschauwer and Hofte, 2007). How similar, with respect to antimicrobial production, is strain (P) to the type strains of *S. plymuthica?* I investigated the similarity of the antimicrobial production of our strain (*S. plymuthica* (P)), and that of two type strains of *S. plymuthica* and the most common *Serratia* spp, in regards to human health (*S. marcescens*). This investigation was carried out to determine whether this marine strain (P) produces inhibitory molecules with different inhibitory activities from the antimicrobial compounds produced by the other *Serratia* spp. Antimicrobial testing (spot test) was carried out as described in Material and Methods (Section 2.1.5), with a type strain of *S. plymuthica,* DSM 4540 and *S. marcescens* (ATCC13880). These experiments revealed that this marine isolate (P) did not produce a similar spectrum of antimicrobial activity to these characterised strains (Table 3.4 and Figure 3.9). However, based on the spot test results (Figure 3.9), the environmental isolate of *S. plymuthica* produced a more uniform antimicrobial clearing zone against the *Bacillus cereus var mycoides test strain* (F) compared with *S. marcescens* (ATCC13880), although both inhibited the growth of this organism, suggesting that the antimicrobial compound(s) produced by P are different. Furthermore, antimicrobial production can be seen when *S. plymuthica* (P) was tested against other bacteria (MRSA and VRE), while *S. marcescens* (ATCC13880) was unable to retard their growth (Table 3.4). Thus, it appears that the antimicrobial compound(s) produced by my environmental isolate of *S. plymuthica* are/ is potentially different than those produced by the *S. plymuthica* DSM 4540 and *S. marcescens* (ATCC13880). These results showed significant phenotypic differences between the marine strain (P) and the other tested *Serratia* spp. Importantly, this supports the likelihood that P is a new strain, or at least is a strain that produces different antimicrobial compound(s).

Table 3.4 Antimicrobial activity of the marine *S. plymuthica* (P), *S. marcescens* ATCC13880 and type strain of *S. plymuthica* DSM 4540 against a variety of microorganisms.

Micro organisms	**Antimicrobial activity**		
	P	***S. marcescens (ATCC13880)***	***S. plymuthica DSM 4540***
Bacillus cereus var mycoides (F)	++	+++	-
VRE	++	-	-
MRSA	+++	-	-
E. coli BW20767	++	-	-

(+++) Very strong antimicrobial activity, (++) strong antimicrobial activity and (-) no antimicrobial activity. Growth condition, 30 ^{o}C /24-NA.



A) B)

Figure 3.9 Antimicrobial activity of marine bacterium *S. plymuthica* P (A) and *S. marcescens* (ATCC13880) (B), on nutrient agar plates with confluent growth of test microorganism *Bacillus cereus var mycoides* test strain (F), grown at 21 ^{o}C for 24hrs.

3.2.4 Quorum sensing bioassay

Studies have suggested that the quorum-sensing system plays a role in the expression of specific genes, including in some instances those for antimicrobial production (Labbate *et al,.* 2004). Several researchers have recently studied the quorum-sensing systems present in *S. plymuthica*. (Labbate *et al,.* 2004; Van Houdt *et al,.* 2007 and Pang *et al*,. 2008). More recently studies performed by Liu *et al*. (2011), have indicated that two QS systems from *S. plymuthica* strain G3 have been characterized and their AHL profiles determined. In contrast, a study was carried out by Van *et al.* (2006) on the *S. plymuthica* strain RVH1 examining the effect of AHLs deletion gene (*luxS* gene knockout) on the production of chitinase, nuclease, extracellular protease, and antibacterial compound and biofilm formation. None of these phenotypes was affected by luxS knockout. As a result an experiment was performed to indirectly determine N-acylhomoserine lactone (AHLs) production using a cross-streaking bioassay as described by Swift et al. (1997). In this assay diffusible AHLs production induces the biosensor strain to produce a purple pigment. It was carried out using the antimicrobial producer *S. plymuthica* (P), *S. marcescens* (ATCC13880), *S. plymuthica* strain DSM 4540 and a terrestrial isolate of *S. plymuthica* at 21 and 30 ^{o}C, against the bio-sensory strain *Chromobacterium violaceum* (CV0blu) (Section 2.1.1.3) on an NA plates. The plates were incubated at (21 ^{o}C and 30 ^{o}C for between 1 and 2 days). A positive result was recorded by the production of a purple pigment in the test bacterial strain *Chromobacterium violaceum* (CV031). The results shown in Figure 3.10 show that both the antimicrobial producer *S. plymuthica* (P) and the other *Serratia* species did not appear to produce diffusible N-acylhomoserine lactones (AHLs).

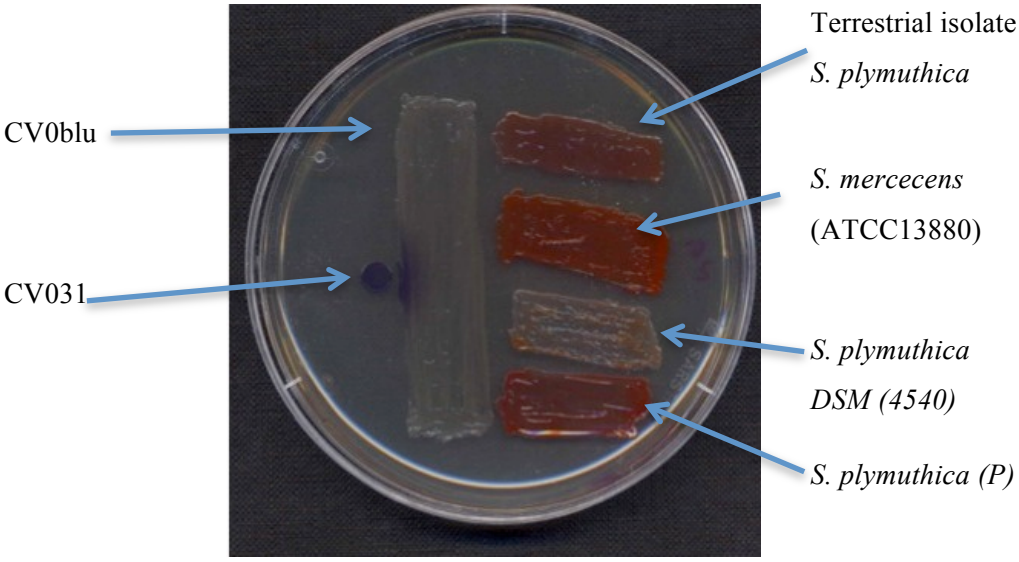


Figure 3.10 AHL production bioassay. Cross streaking of *S. plymuthica* (P), *S. marcescens* (ATCC13880), *S. plymuthica* strain DSM 4540 and a terrestrial isolate of *S. plymuthica* (right), against biosensor strain *C. violaceum* (CV0blu) (centre) and *Chromobacterium violaceum* (CV031) (Positive control), grown at 30 ^{o}C for 24-48hrs.

4. Isolation and preliminary characterisation of mutants unable to produce the antimicrobial (s)

4.1 Introduction

In order to gain an insight into the type of antimicrobial compound(s) produced by strain P a genetic approach was used in an attempt to identify the gene(s) involved in antimicrobial production. In this study, fourteen mutants, unable to produce antimicrobial compound(s) have now been obtained using transposon mutagenesis. The isolation, preliminary identification and characterisation of the gene(s) involving the antimicrobial production are presented here.

4.2 Results

4.2.1 Tn*5* mutagenesis

It has been recently reported that transposon delivery vector pRL27 is efficient, useful and functional for the genetic analysis of Gamma-Proteobacteria such as *E. coli*, *Salmonella enterica* serovar typhi, *Vibrio cholerae* El Tor C6706 (Larsen *et al*., 2002). Therefore, it was decided to use Tn*5* mutagenesis in an attempt to obtain non-antimicrobial mutant strains, from the marine isolate strains (P). To achieve this a transposon mutagenesis technique was performed, using the system designed by Larsen *et a*l., (2002). *S. plymuthica* (P), was used as a recipient, in conjugation experiments with the *E. coli* donor strain (BW20767 carrying the Tn*5*-pRL27 ((Km^R-*ori*R6 K)) plasmid (Materials and Methods, Figure 2.4). A flow chart of the procedures used in the conjugation experiments is shown in Figure 2.5 (Materials and methods). Screening for potential mutants was carried out using nutrient agar plates containing two antibiotics (20µg/ml Ap, and 50µg/ml Kan). Figure 2.6 (Materials and Methods) describes the

method used in this study to screen for potential mutant strains (PM). Following screening for *S*. *plymuthica* carrying an integrated Tn*5*, it became evident that the transposon mutagenesis was successful; with frequency for recipient cells (about $9.1\pm0.2\times10^{-4}$ CFU ml^{-1}). Approximately 5000 transposon insertion mutants were screened in this study (Figure 4.1). Those mutants that were unable to produce a growth inhibition zone against sensitive strain *B. cereus var mycodies* (F); were considered as potential mutants and colony purified (Figure 4.2). Following re-testing, 14 non-antimicrobial producing mutant strains were obtained (Figures 4.3) numbered as follows: 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 19

One of these mutants was non-pigmented (4) whereas the other thirteen are pigmented (Figures 4.4)

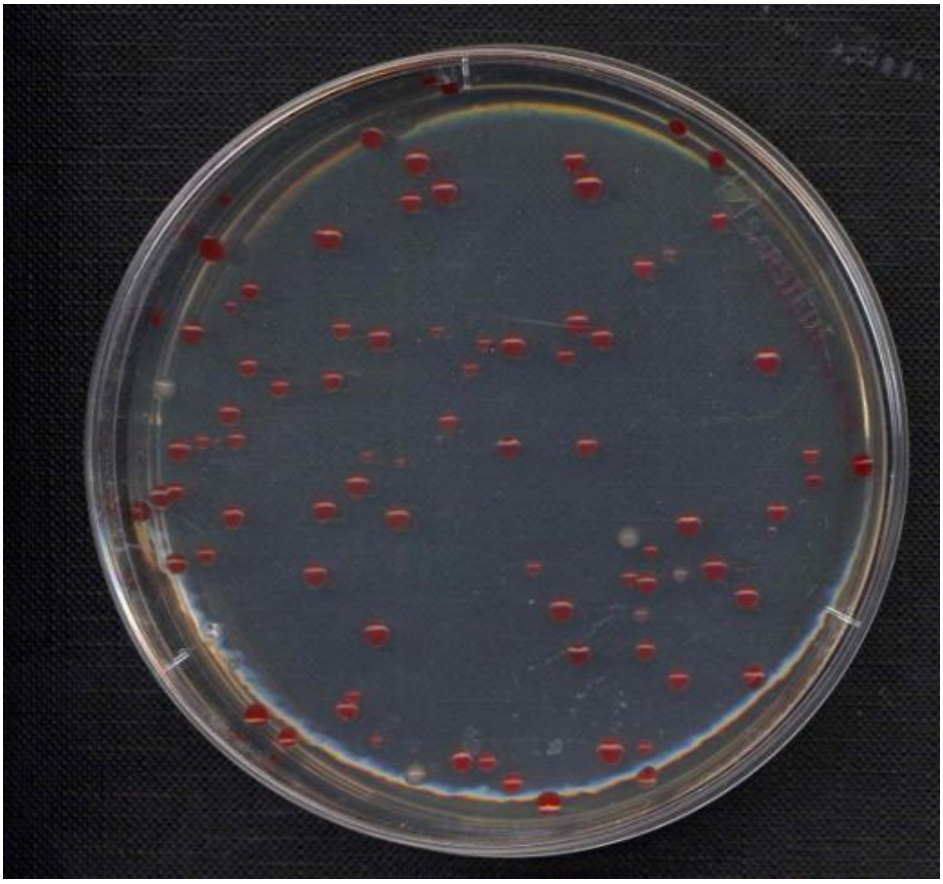


Figure 4.1 A plate showing antibiotic-resistant transconjugants of marine isolate *S. plymuthica* (P) colonies growing on Nutrient agar + 50µg/ml Kanamycin (Kan) +20µg/ml Ampicillin (Amp).

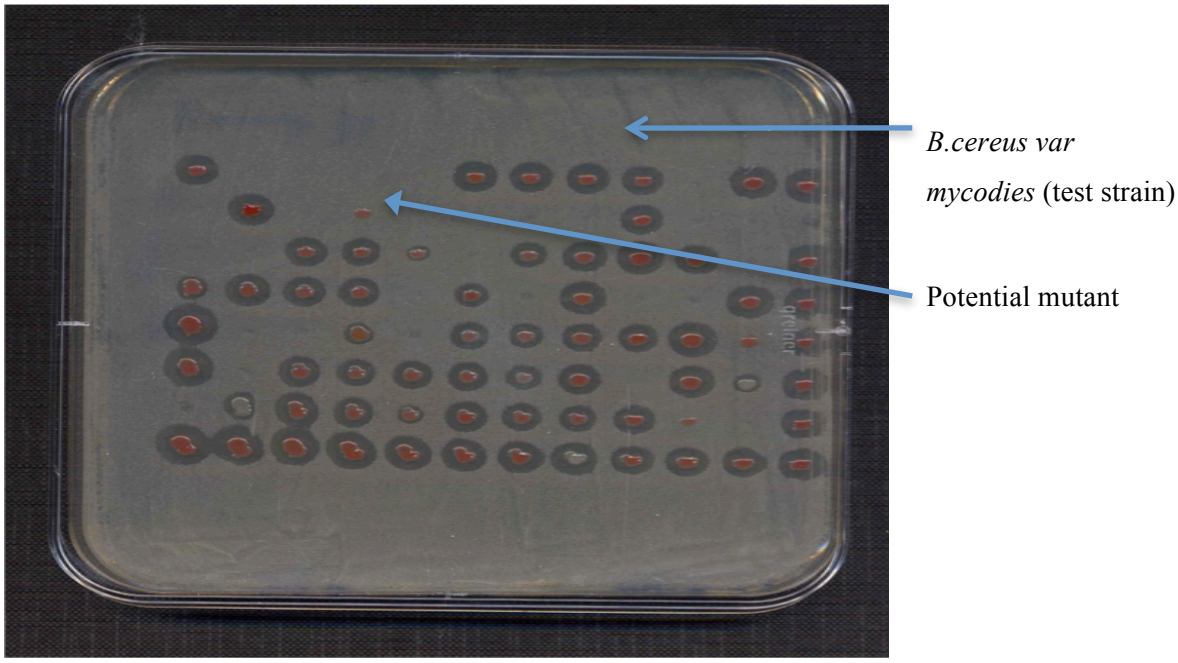


Figure 4.2 Screening for potential non-antimicrobial mutant strains, indicator micoorganism; *B. cereus var mycodies* (NA plate after 24hour incubation at 21ºC).

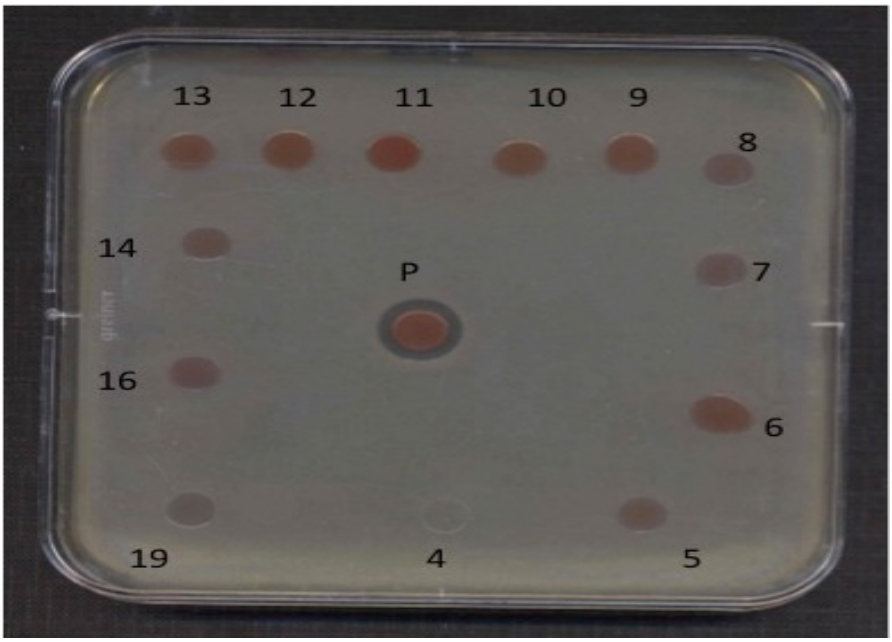


Figure 4.3 A plate showing the lack of antimicrobial production phenotype of the transposon mutants, with test microorganism *B. cereus var mycodies* (N-A plate after 24hour incubation at 21ºC).

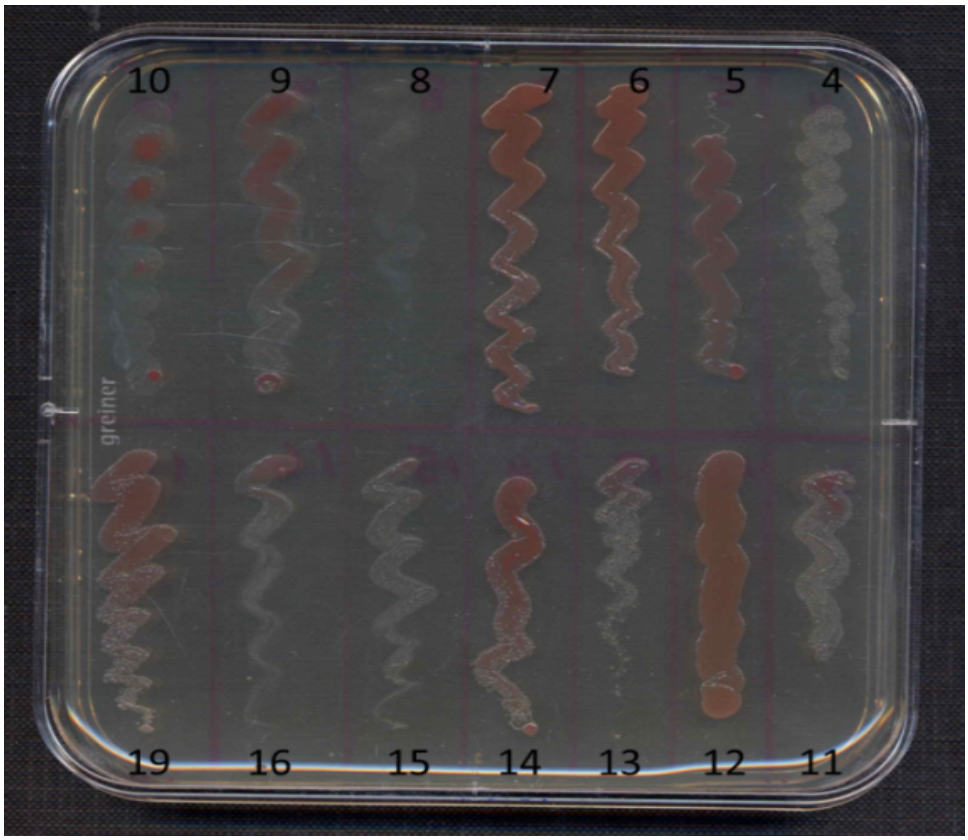


Figure 4.4 A plate showing the non-antimicrobial producing mutants growing on Nutrient agar + 50µg/ml Kan + 20µg/ml Amp to confirm they were all resistant to Kanamycin as well as Ampicillin (24hrs incubation at 21ºC). One of these mutants is non- pigmented (No 4).

4.2.2 Ensuring that mutants result from the insertion of the transposon into the genome of strain P

Since the Km^R gene is within the transposon element, a PCR reaction was carried out to ensure that the Km^R phenotype of the mutants resulted from the insertion of the transposon into the *S. plymuthica* genome. A PCR reaction was performed (Section 2.2.6) using specific primers (AGPT-F and AGPT-R, {Table 2.7, Materials and Methods}) using genomic DNA as a template in the reaction.

Amplified DNA from the PCR reaction was then subjected to gel electrophoresis and the results show a band in each of the potential mutants, corresponding in size (600 bp) to the

positive control (amplified from the pRL27 plasmid) (Figure 4.5). Therefore, these strains carry the transposon (the Kanamycin resistant (Km^R) gene) within their genomic DNA.

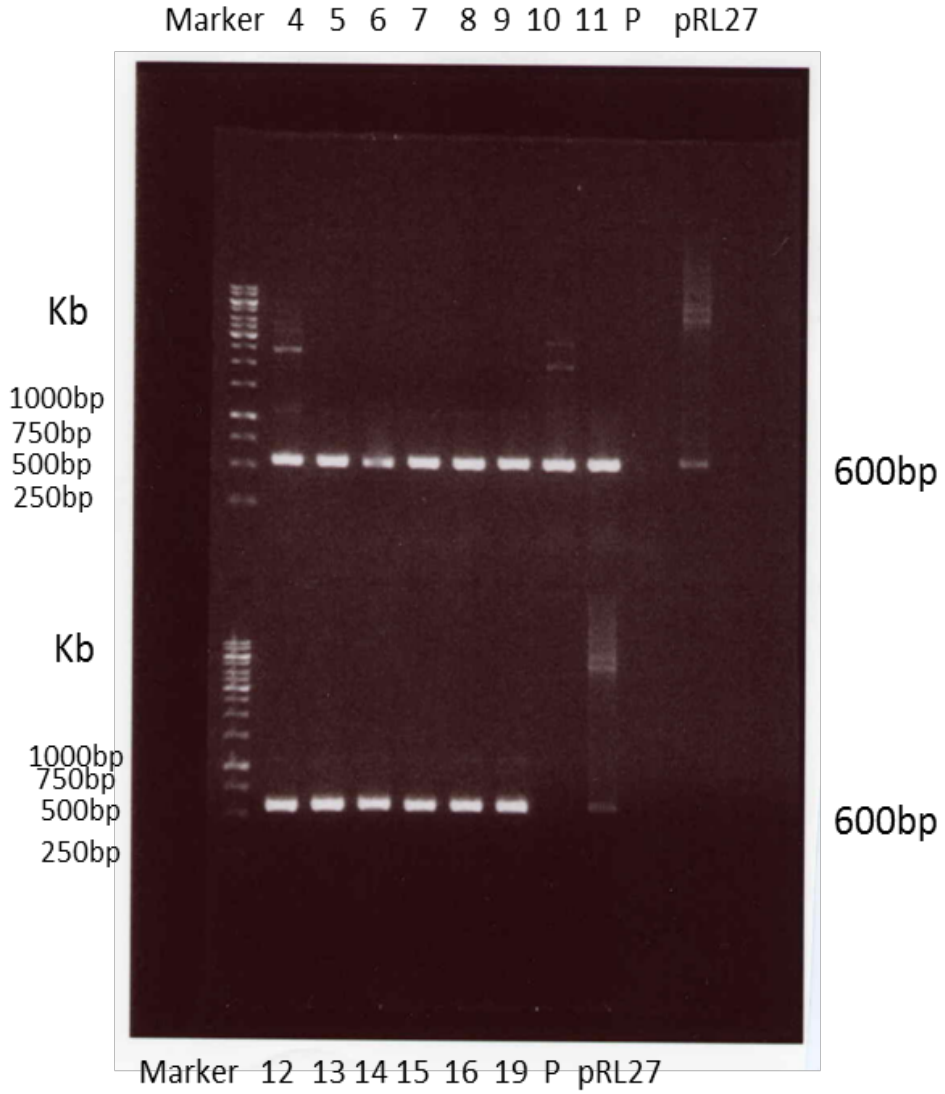


Figure 4.5 Agarose gel electrophoresis of the PCR amplified Kanamycin resistance gene (Km^R) from the non-antimicrobial producing mutants. Aliquots, 5µl, of each PCR reaction were run on an 0.7% agarose gel and DNA was detected using ethidium bromide staining. Lane Marker: 1 kb ladder, lane 4-19: PCR products from non-antimicrobial producing mutants 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 19 respectively, lane P: Negative control PCR reaction using wild type *S.plymuthica*, P genomic DNA as template and lane pRL27: Positive control (PCR reaction using pRL27 as a template).

4.2.3 Characterisation of the transposon mutants

It was important to initially determine whether or not the isolated mutants were the result of single or multiple transposon insertions. Furthermore, determination of the size of the *Bam*HI genomic DNA fragment that the transposon had been inserted into should help to provide an insight how many different genes may be involved. Therefore, southern blot analysis was used to obtain physical evidence of the number of transposon insertion sites in these mutants. The chromosomal DNA of the mutants (4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 19), was digested with *BamHI*, which does not cut within the transposon. (Section 2.2.4, Materials and Methods) (See Figure 4.6 for *Bam*HI digested genomic DNA), and then hybridised with the Km-DNA probe (labelled with DIG), as described in Sections 2.2.11 and 2.2.12 (Materials and Methods). The results shown in Figure 4.7 demonstrate that single transposons are independently integrated within the chromosomal DNAs of the selected mutant (one band only in each lane). Therefore, the southern blot results indicate that the fourteen insertions of each of the test-selected mutants were present in the genomic DNA, and in the single copy. Furthermore, the size of the *Bam*HI genomic DNA fragment that the transposon had been inserted into differs depending on each mutant. Fragment sizes were determined as shown in Table 4.1.

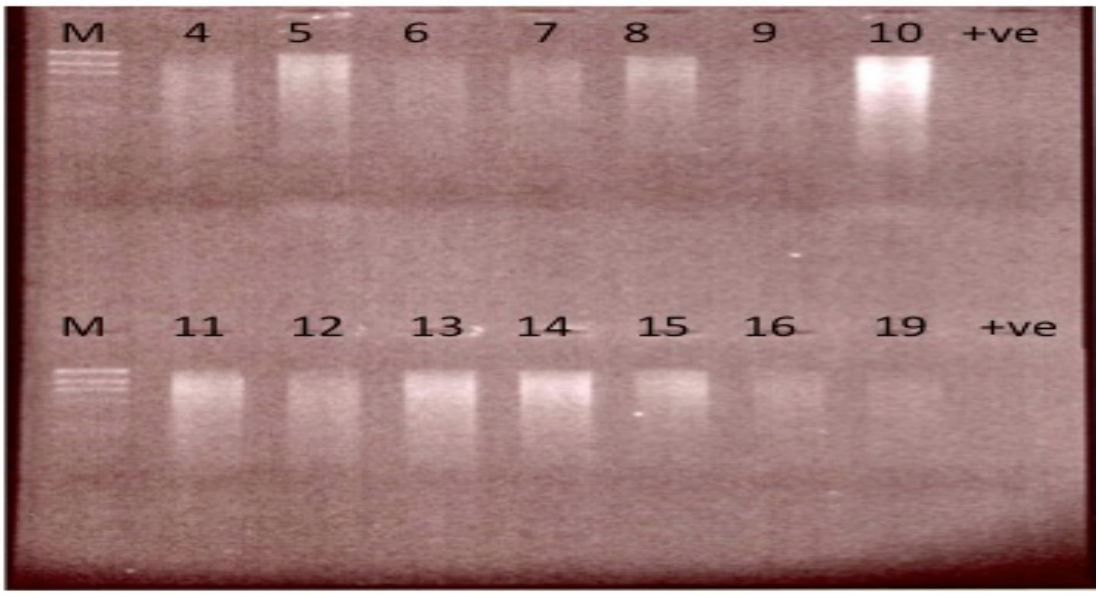


Figure 4.6 The agarose gel electrophoresis of *Bam*HI digested genomic DNA. M: DIG labelled marker (λ *Hin*dIII), 4-19: *Bam*H1cut genomic DNA from mutant No. 4-19, respectively. +Ve: Unlabelled purified PCR Km^R gene from PRL27 plasmid (positive control, 1/30 fold dilution).

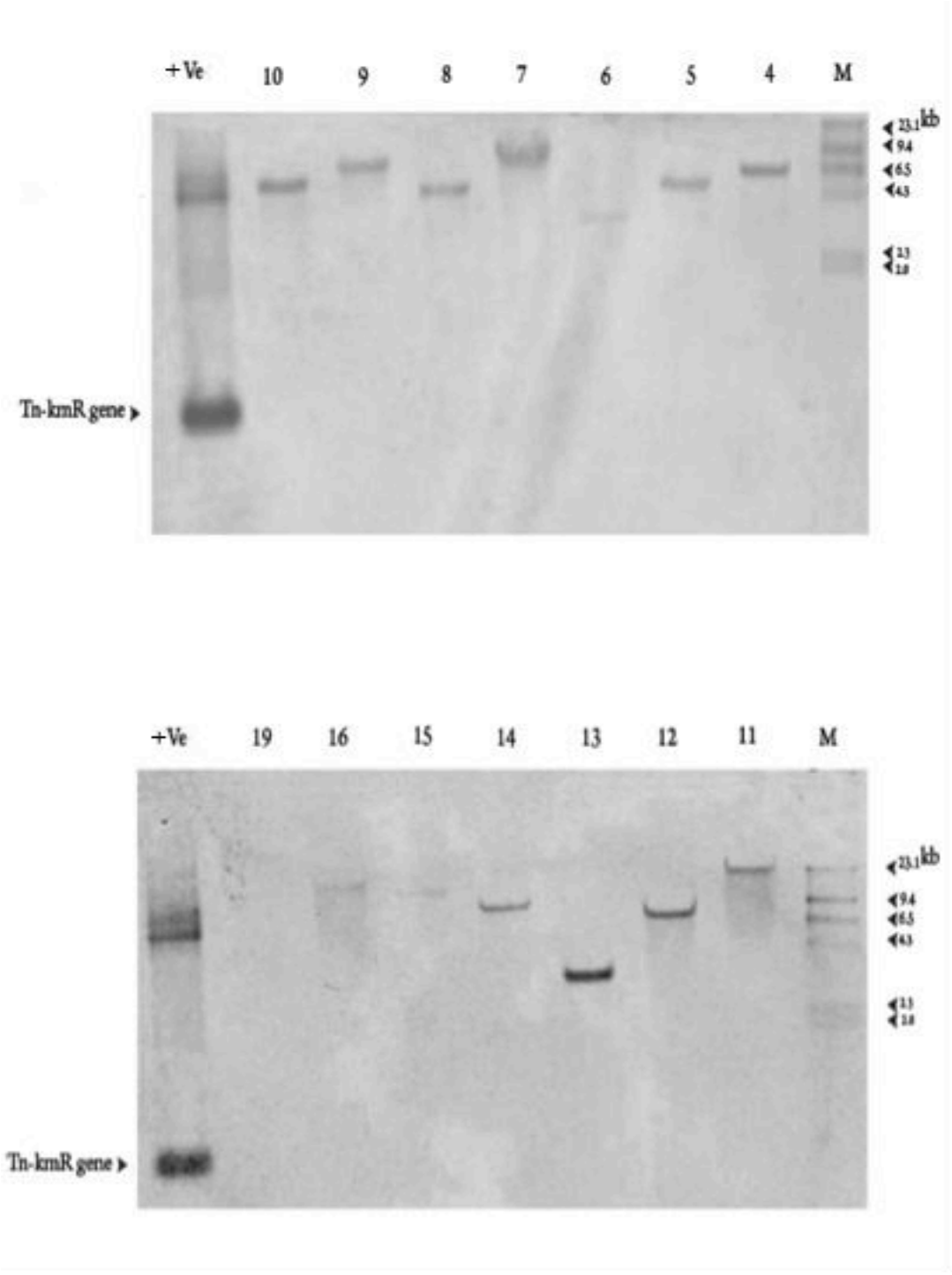


Figure 4.7 Southern blot hybridisation with *Bam*HI, digested DNA prepared from mutants (Nos. 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 19). The 0.6- kb DIG labelled purified PCR Km^R gene from pRL27 plasmid was used as a DNA probe. M: DIG labelled marker (*Hind*111) digested DNA marker, +Ve: Unlabelled purified PCR Km^R gene from the pRL27 plasmid (positive control), 4-19: *Bam*H1 cut genomic DNA from mutant Nos. 4-19, respectively.

Table 4.1 The size of the transposon containing *Bam*HI fragments in the mutants, as determined by Southern blot analysis.

Mutants No.	Fragment sizes (Kb)	Fragment sizes (Kb) without the transposon (1.8 Kb)
4	6.5	4.7
5	5.7	3.9
6	3.1	1.3
7	9	7.2
8	5.7	3.9
9	6.5	4.7
10	5.7	3.9
11	22	18.2
12	6.5	4.7
13	3.5	1.7
14	6.9	5.1
15	12.8	11
16	15	13.2
19	12.8	11

4.2.4 Cloning and recovery of the transposon insertion and flanking genomic DNA

Having isolated transposon insertion mutants no longer producing antimicrobial activity, the next stage was to identify the gene(s) defined by these insertions. This would involve the cloning and recovery of the transposon insertion and flanking genomic DNA from each mutant. Genomic DNA from the mutants was extracted (Section 2.2.1), and then digested with *Bam*HI, which does not cut within the transposon (Section 2.2.4). The digested chromosomal DNA was then ligated (Section 2.2.5) and introduced into chemically competent *E. coli,* strain S17-1 λ*pir* (Section 2.2.13). The transformation mixes were then plated onto selective LB agar plates containing Kan (50µg/ml) and Str (30µg/ml) and incubated overnight at 37ºC. Figure 2.7 (Materials and Methods) shows a diagram summarising the cloning, recovery and sequencing of the resulting transposon junction plasmids (TJP). The plasmids were purified as described in Section 2.2.2 and

subjected to sequencing as described in Section 2.2.9 (Materials and Methods) with two primers tpnRL17–1 and tpnRL13–2 (Table 2.7), which anneal to the ends of the transposon (oriR6K and Km^R, respectively) (For primer sites, see Materials and Methods; Figures 2.8). This approach was successful for mutants 4, 5, 7 and 8 respectively. The analysis of these plasmids by agarose gel electrophoresis (Figure 4.8) shows that the sizes of these particular TJP were varied; TJP from mutant No. 4 was (8.0 Kb), TJP from mutant No. 5 (5.0 Kb), TJP from mutant No. 7 (8.0 Kb), and TJP from mutant No. 8 (6.0 Kb). All of these TJP (4, 5, 7 and 8) were cut with unique restriction enzyme with *Bam*H1 to form linearised DNA. However, attempts to clone the transposon insertion mutants from the following mutants (6, 9, 10, 11, 12, 13, 14, 15, 16 and 19) were not successful despite repeated attempts. It is unknown why the other 10 mutants failed to clone during the cloning stage. A potential explanation for this could be that result from issues with chemically competent *E.coli* cells. For example, they weren't competent enough in order to receive the recombinant DNA or that they were damaged during the treatment for making them competent or that the transposon junction plasmid (TJP) DNA, transferred into the competent *E.coli* cells, then integrated with competent chromosomal DNA.

The genomic DNA flanking on the transposon insertions for the mutants (4, 5, 7 and 8) were partially sequenced, as described in the material and methods, chapter 2 (Figure 2.7). Then the DNA sequence obtained was compared with the sequences in GenBank using the BLAST program. Comparison of the resulting sequences against the genomes for *S. marcescens* and *S. plymuthica* (BLASTN), revealed that is no homology at the DNA level. Moreover, none of the four-transposon junction plasmid sequences obtained (4, 5, 7 and 8) were found to be related to the delivery vector. Nevertheless, comparison of the resulting sequences (BLASTX) revealed that the four mutants (4, 5, 7 and 8) had insertions into gene(s) that were similar to those found in the other *Serratia* spp. when encoding non-ribosomal peptide polyketide synthases (Figure 4.9). This result indicates that the levels of matching, are not significantly high; for instance, they were (31%) according to the partial sequencing of the flanking genomic DNA from mutant No 5 (Figure 4.10). (More blast results are shown in Appendix 2).

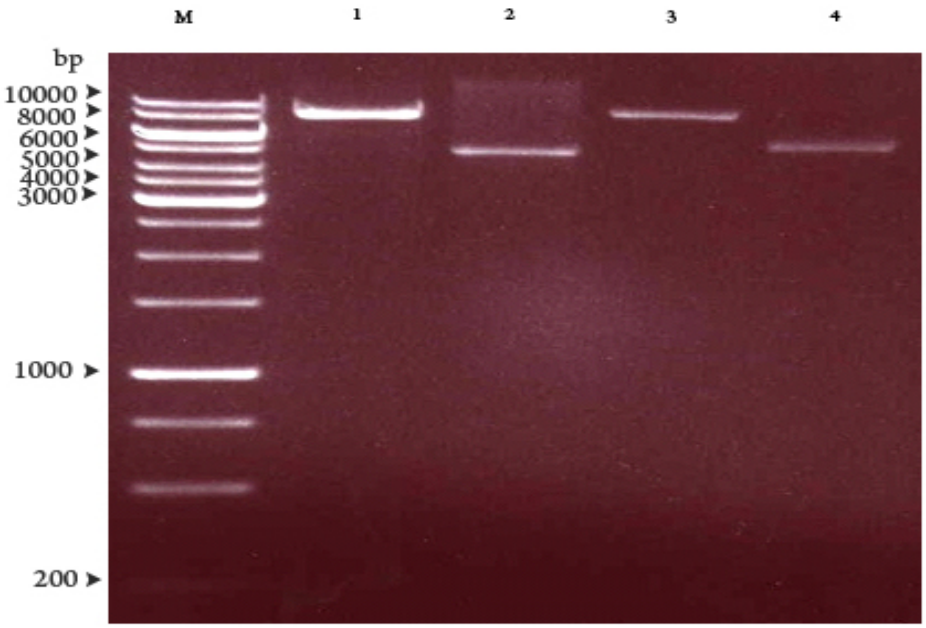


Figure 4.8 Gel electrophoresis of *Bam*HI digested transposon junction plasmids (minipreps). Briefly, 5µl of *Bam*HI digested mini-prep was run on a 0.7% Agarose gel and stained with ethidium bromide. Lane M: 1 kb ladder, lane 1: TJP from mutant No 4, lane 2: TJP from mutant No 5, lane 3: TJP from mutant No 7, and lane 4: TJP from mutant No 8.

Description	Max score	Total score	Query cover	E value	Max ident	Accession
polyketide synthase [Serratia plymuthica]	72.8	135	11%	1e-13	50%	gi|417353285|AFX60332.1
polyketide synthase [Serratia plymuthica]	71.2	253	12%	4e-13	49%	gi|417353289|AFX60336.1
polyketide synthase [Serratia plymuthica]	69.7	119	22%	1e-12	49%	gi|417353287|AFX60334.1
polyketide synthase [Serratia plymuthica]	66.2	66.2	10%	1e-11	44%	gi|417353293|AFX60340.1
polyketide synthase [Serratia plymuthica]	93.2	239	22%	5e-20	43%	gi|417353294|AFX60341.1

Figure 4.9 The blastx search result obtained from the partial sequencing the flanking genomic DNA from mutant No 5, showing that transposon had inserted into a gene, with similarity to a gene encoding a Polyketide synthesis.

```
Query  456   FEXTNIKALAEHLEENFDISELVESTKQPKAHVSINKKEHN--------SFVSGSMKEEL  301
             ++  NI+ALAE++    D      +T +PK + +++  E          + V+ +  EE
Sbjct  1069  YDYPNIQALAEYIASQSD-----RATPRPKPNAAVSAPEDACPEVLKPAASVALARSEEA  1123

Query  300   VTNNDGKLQSSDIAVIGMSGRYPQSSNLSEFWENLKAAKDCITEVPSDRWDSSK-FDNFV  124
               ++    +   IA+IGM+GRYP + NL+++W+NL   ++ + EVP  RWD  + FD
Sbjct  1124  AISHR---RQEKIAIIGMAGRYPDADNLAQYWDNLANGRNAVREVPKARWDVDQYFDADR  1180

Query  123   SPSGKKMSKWGG  88
               GK   KW G
Sbjct  1181  RAEGKVYCKWLG  1192
```

Figure 4.10 Amino acid sequence line-ups (Blastx) between *Serratia plymutica* (P) flanking end of transposon insertion site, mutant 5, sequence from primer 13-2(Query) and Polyketide synthesis, from *Serratia plymuthica* (Sbjct). The E-value for this match is 1e-12, and the sequence shows 31% identities and 52% similarities.

4.2.5 Sequencing the flanking regions

An attempt to sequences the flanking regions was carried out in this study to help understand is the structure of the genes involved e.g. size of the open reading frame (ORF), is it a single transcription unit or part of an operon, are there any readily identifiable transcription control sequences that could give an insight to the control of antimicrobial production? In order to sequence the whole flanking regions of the transposon junction plasmids (TJPs), the sequences obtained using the outward directed sequencing primers (Previous Section {4.2.4}), was used to design primers to extend the sequence coverage, in an attempt to extend the sequence (Table 2.7). In this study I

have partially sequenced the genomic DNA flanking the inserted transposon in mutants 5, 7 and 8 (See Appendix 1 for sequence data). Based on the sequencing data obtained from this part of study, it was decided that it would be worthwhile to attempt to clone the genomic DNA region containing the antimicrobial gene(s) (*ang*).

4.2.6 Attempt to clone the genomic DNA region containing the antimicrobial gene(s) (*ang*)

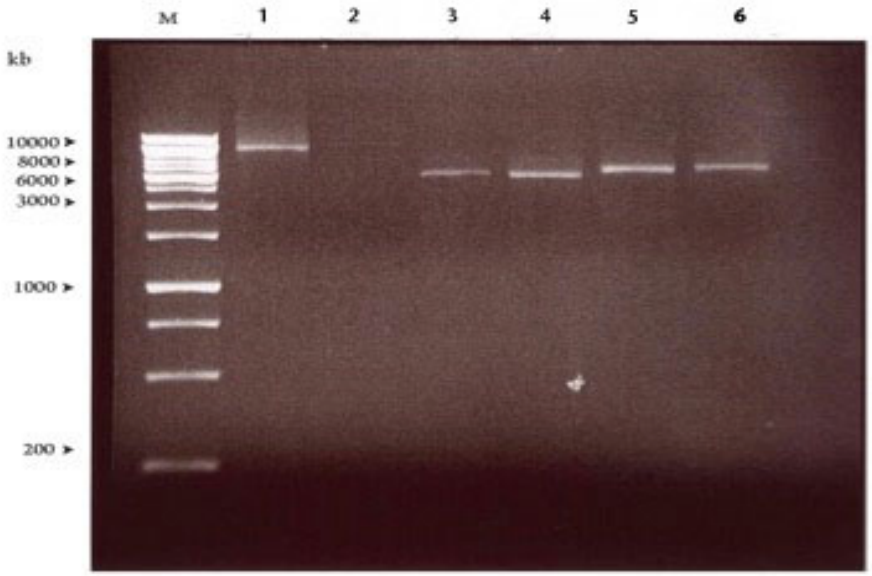
Further genetic approaches were conducted to gain an insight into the gene(s) required for the synthesis of the antimicrobials such as cloning into a plasmid vector. I attempted to clone the *ang* with the aim of establishing a first step for further study, to allow the *ang* to be studied in greater detail for purposes such as gene characterisation (regulation and mapping). A gene cloning method was carried out using primers designed for a Long PCR reaction, as shown below:

4.2.6.1 PCR amplification of genomic DNA defining the antimicrobial gene(s)

The flanking region sequencing results (Section 4.2.5 and Appendix 1), from the non-antimicrobial mutant strains (M 5, 7 and 8), were used to design specific primers for the amplification of the gene(s) (bold line in Table 2.7, Materials and Methods). These primers would result in products containing *Bam*HI sites at each end, enabling easy cloning. After designing the primers, genomic DNA from strain P, was isolated (Section 2.2.1, Materials and Methods) and used as a template, in a long PCR reaction as described in Section 2.2.6 (Materials and Methods). The products from the PCR were subjected to gel electrophoresis (Section 2.2.8, Materials and Methods) and the results obtained showed that the product of mutant's No. 5 and No. 8 yielded a DNA fragment of ~5 to 6 kbp; whereas, mutant No. 7 produced a fragment of around 9 kbp (Figures 4.11). In comparison, this result is in agreement with the results obtained from the southern blotting and gel electrophoresis of cloned insertion mutants (TJPs) mini-prep experiments (Table 4.1 and Figure 4.7). Afterwards, the long PCR products were then subjected to gene cloning as described in the next Section.

4.2.6.2 Cloning of the *ang*

After checking that the target DNA fragment was successfully amplified, using gel electrophoresis (Section 2.2.8), attempts were made to clone the PCR products (from mutant's No. 5, No. 7 and No. 8) into a DNA cloning vector, pBluescript II KS (-)(Figure 2.2; Materials and Methods). Amplified PCR products were cleaned using a PCR Purification Kit (Qiagen), then digested with *Bam*HI (Section 2.2.4, Materials and Methods), and ligated into *Bam*HI cut pBSII KS (-) (Section 2.2.5, Materials and Methods) and introduced into chemically competent *E. coli* XL1-Blue cells (Sections 2.2.13, Materials and Methods). Afterwards, screening for successful transformants was completed using a blue/white colour screen. Briefly, the transformed cells were plated out on LB plates containing 50 µg/ml Ap and 40µl of 0.1 M Isopropyl thioglactoside (IPTG) and 2% 5-bromo-4-indolyl-beta –D- galactopyranoside (Xgal) that was spread on top of the LB plates and left to dry before plating, plates were incubated aerobically at 37 ^{o}C for 18-24hrs. Following the period of incubation, the white colonies should be successful transformants (recombinant DNA; plasmids with inserts), whilst blue colonies will be plasmids without inserts. Despite multiple repeats of the experiment, using different competent cells, no plasmids containing the desired insert were isolated. The precise reason why my cloning experiments were unsuccessful is unknown; the reason, why I could not get constructed plasmid DNA is unknown, it is possible that the cloned gene was transcribed and translated, and the yielded a protein that was toxic to *E. coli*. In general, cloning genes involving antimicrobial production is usually more challenging than cloning other genes (Martín and Gil, 1984).



Figures 4.11 Analysis of the long PCR products from mutants 4, 5 and 8. Lane M: 1 kb ladder, lane 1: amplified DNA using designed primers from mutant No. 7, lane 2: amplified DNA cut with *Bam*H1. Lane 3: amplified DNA using designed primers from mutant No. 5, lane 4 amplified DNA cut with *Bam*H1, lane 5: amplified DNA using designed primers from mutant No. 8, lane 6: amplified DNA cut with *Bam*H1. (0.7% Agarose gel).

5. Characterisation of the antimicrobial agent(s)

5.1 Introduction

Several studies have indicated that antimicrobial compounds produced by terrestrial *S. plymuthica* strains can be associated with chitinolytic enzymes or 1,3- glucanases (cell wall degradation enzymes) (Berg *et al*., 2002; Frankowski *et al*., 1998; Kamensky *et al*., 2003; Kurze *et al*., 2001); or antibiotics such as antibiotic delivery (siderophores) and pyrrolnitrin (Kamensky *et al*., 2003; Meziane *et al*., 2006; Calvente *et al.,* 2001). However, most work conducted on *S. plymuthica* strains is in the area of soil microbiology and biocontrol (Compant *et al*., 2005; Meziane *et al*., 2006). In this study, the producer strain (P) was obtained from the marine environment; consequently, different strains of *Serratia* might produce different types of inhibitory molecules. This was clearly observed from comparisons between the isolated strain P and both the type strain (*S. plymuthica* DSM 4540) and the most common *Serratia* spp in regards to human health (*S. marcescens* ATCC13880). The results showed that the molecule(s) produced by P had a different antimicrobial spectrum. Therefore, these results showed significant phenotyping differences between the marine isolate strain (P), and the other tested species, *Serratia*. Importantly, this finding supports the hypothesis that the marine environment isolate *S. plymuthica* (P) is potentially a new strain, or at least is a strain that produces different antimicrobial compound(s). In view of this, it was worthwhile to attempt identification of the inhibitory molecule (s) produced by strain P.

5.2 Results

5.2.1 The effect of different cultivation conditions on the production of the antimicrobial compound(s)

A range of different cultivation conditions were employed in an attempt to produce higher levels of antimicrobial compound from the marine isolate P. In these experiments, bacterial cultures were incubated at 21^{o}C while shaking at 220 r.p.m for 24hrs, in a variety of growth conditions. These included different surface attachment materials, different growth media, and use of roller bottle (rb) culture. In addition the effect of cross-species interaction, following exposure to a sensitive strain *Bacillus cereus var mycoides* (F) was also examined. The antimicrobial activity was tested using disc and spot tests (Materials and Methods, Section 2.1.5) against *Bacillus cereus var mycoides* on NA plates.

5.2.1.1 The effect of growth media on antimicrobial production

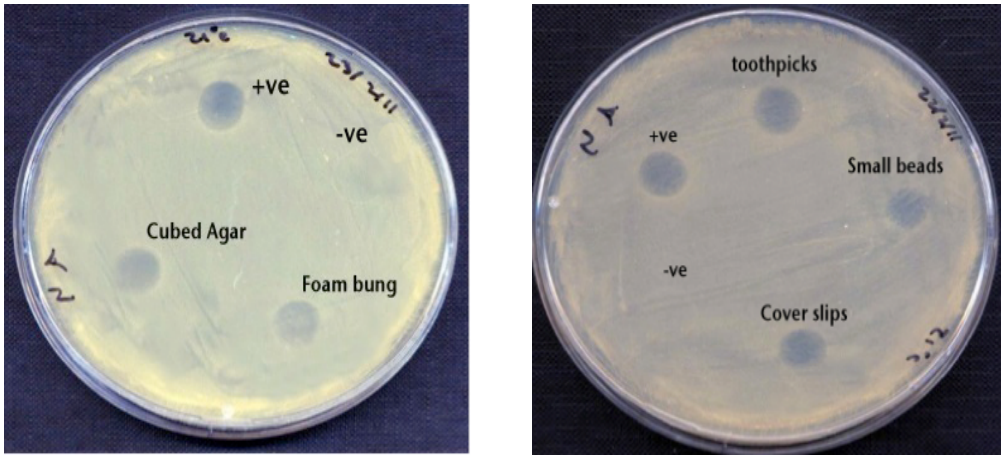
Several studies have demonstrated that the chemical composition of growth media significantly affects antimicrobial activity (Martin and Demain, 1980) (Farmer, 1985) (Spížek and Tichý, 1995) (Marwick *et al*., 1999). Therefore, it was decided that it was worthwhile to cultivate the producer strain P in different growth media to study its effects on the antimicrobial compound production. 250ml conical flasks containing 100 ml broth media; Nutrient broth (NB), Brain heart infusion broth (BHI), Luria Bertani broth (LB), Tryptone soya broth (TSB) or Marine broth (MB), were inoculated with 100µl of an overnight culture of strain P. The flasks were then incubated aerobically at 21^{o}C with shaking (220 r.p.m.) for 24hrs. After the period of incubation, bacterial cultures (100 ml) were spun at 13000 rpm for 10 minutes using a large centrifuge (Beckman Avanti J-26 XP). The cell pellet was then discarded and the spent culture medium (termed as SM) was filter sterilised using a 0.2µm filter (Millipore) and stored in the refrigerator (9 ^{o}C) until used. The antimicrobial activity of the spent culture medium (SM) was then assessed using a spot test (Materials and Methods, Section 2.1.5) using *Bacillus cereus var mycoides* (F) as the sensitive strain. The results showed that there was a decrease in the

antimicrobial activity of the SM from LB and MB media in comparison with the SM from TSB, BHI and NB grown cultures (Figure 5.1). An explanation for this could be that the presence of the salt (sodium chloride) in the LB and MB media seems to suppress inhibitory activity. However, this result is in agreement with the results obtained by evaluating an inhibitory activity of the live cultures of strain P grown on different agar media which showed that the inhibition zone around the culture drop was much wider on the nutrient agar plates when compared with the other media tested (LB and TSA and BHI) (Section 3.2.2.4, Figure 3.5). In conclusion, it is evident that the chemical composition of growth media significantly affects antimicrobial activity and unfortunately the use of different growth media failed to enhance the antimicrobial production from strain P.

Figure 5.1 Effect of different growth media on antimicrobial production. 20µl of spent media (SM) from cultures of P grown in different broth media was spotted onto a lawn of *Bacillus cereus var mycoides* (F) on NA plates, 21 °C /24hrs. The spent culture media were obtained from cultures grown in following media: Nutrient broth (NB), Brain Heart Infusion broth (BHI), Luria Bertani broth (LB), Tryptone Soya broth (TSB) and Marine broth (MB). Sterile H_2O were used as negative control.

5.2.1.2 The effect of different surface attachment substrates on antimicrobial production

A variety of surface attachment materials and modified roller bottle (rb) cultures were used with the aim of stimulating the physical condition of the marine bacteria when grown in the marine environment, on the surface of a marine plant (seaweed). Several studies have reported that using different surface attachment substrates enhances the antimicrobial activity. For instance, Yan et al, reported that using these culture conditions leads to the induction of antibacterial production isolated from marine environment (Yan *et al*., 2003). Therefore, an attempt was made to produce higher levels of antimicrobial compound(s) from strain P in liquid cultures by using different surface attachment substrates. Strain P, was inoculated in 100 ml flasks containing sterile 50 ml NB media, which contained different materials; wood (tooth picks), glass (cover-slips), plastic washing-up sponges (approximately 1 cm^3 foam bungs) and small beads with holes. The growth media (6 flasks including a control (NB without material) was prepared separately for each of the materials that were tested. The flasks were then incubated aerobically at 21^oC with shaking (220 r.p.m.) for 24hrs. After the period of incubation, the spent media from each flask was treated as highlighted previously to determine the antimicrobial activity present (Section 5.2.1.1). The results showed no enhancement on the production of the inhibitory molecules under the experimental conditions in comparison to the control (NB without material) (Figure 5.2).



A) B)

Figure 5.2 The effect of growth on a solid substrate on antimicrobial production. 20µl of spent media (SM) from cultures of P with different surface attachment materials was spotted onto a lawn of *Bacillus cereus var mycoides* (F) as sensitive strains on NA plates, incubated at 21 ^{o}C for 24hrs. **A:** Foam bung and cubed agar. **B:** Cover slips, small beads and toothpicks. Sterile Nutrient broth (-VE control) and spent media from cultures of P in NB without material (+Ve control).

5.2.1.3 The effect of modified roller bottle (rb) cultures on antimicrobial production

Further attempts were also performed at this stage of the study to enhance the production of the antimicrobials using modified roller bottle (rb) cultures. Briefly, a 500 ml Duran bottle-containing freshly poured hot 100 ml sterile NA was rolled in ice (3-5 mins) to create a thin layer of NA agar around the inner bottle surface (to simulate the physical condition of the strain P growing in the marine environment, on the surface of a seaweed. Following this, 50 ml of sterile NB was added in addition to 100 µl of an overnight culture of strain P. The bottle culture was then incubated on a roller at high speed (220 r.p.m, 21^{o}C for 24hrs). The antimicrobial activity was then assessed as previously described (Section 5.2.1.1). Kanamycin (50µg/ml) was used as a positive control in spot and disc tests. The antimicrobial spot results showed that the use of modified roller bottle (rb) cultures failed to enhance the antimicrobial production from the marine isolate P in comparison to the control an NB shake flask culture (Figure 5.3).

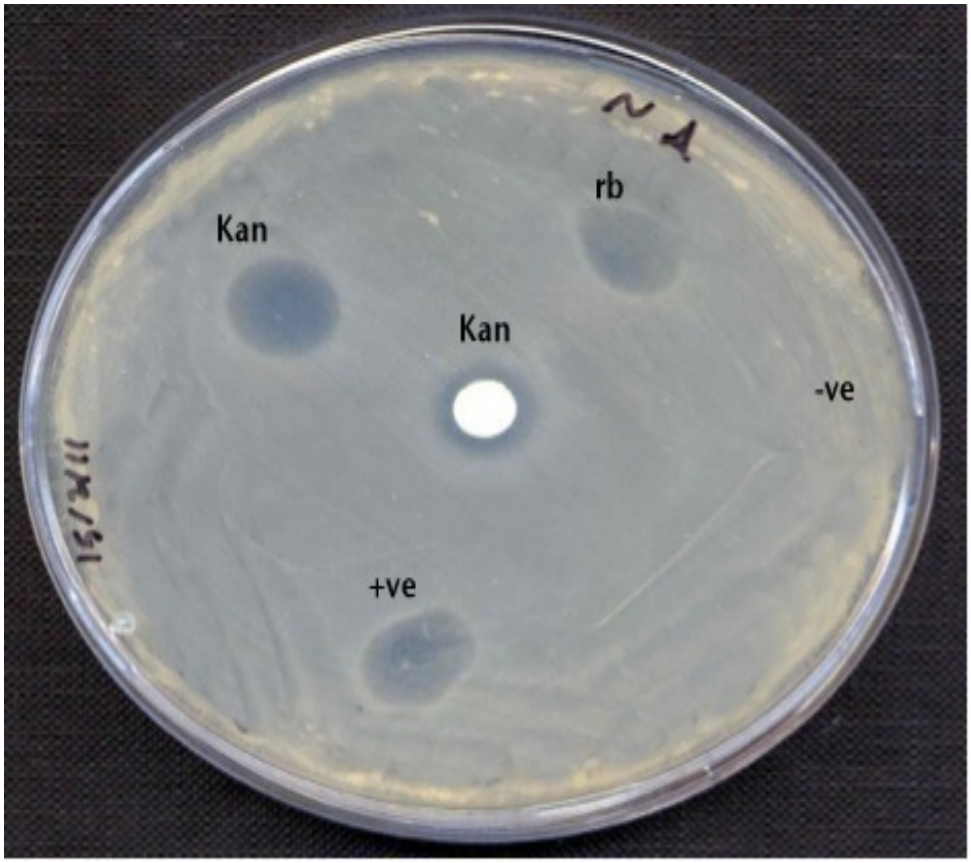


Figure 5.3 Effect of growth on an agar surface on antimicrobial production. 20µl of spent media (SM) from modified roller bottle (rb) cultures were spotted onto a lawn of *Bacillus cereus var mycoides* (F) on NA plates, incubated at 21 ^{o}C for 24hrs. Modified roller bottle (rb), Kan= Kanamycin (20µl of a 50µg/ml solution) and spent media from cultures of P in NB (+Ve control).

5.2.1.4 Does the presence of a sensitive strain enhance antimicrobial production?

One possibility was that the production of antimicrobial activity could possibly be induced by the presence of a potential sensitive bacterial strain. As a result a further attempt was made to produce higher levels of antimicrobial compounds from the marine isolate P in a shake flask culture to which a sensitive bacterial strain was also grown. Two separate experiments were performed; one using heat-killed cells and another using live

cells of the sensitive bacterial strain. A culture of the marine isolated sensitive strain *Bacillus cereus var mycoides* (F) was used, both as heat-killed cells (90^{o}C for 10minutes) and live cells. Briefly, either a live culture (2 ml), a heat-killed culture of *Bacillus cereus*

var mycoides (F) (2 ml), SM from a culture of F (2 ml) or NB media (2 ml) was placed into a 5 cm length of sterile dialysis tubing, to prevent the direct interaction between the sensitive strain and the antimicrobial producer, but allow the free diffusion of any key small molecules. The tubing was then sealed using sterile clips, and each piece of tubing placed into separate 500 ml flasks containing 100ml of sterile NB media. Following this, an overnight culture of strain P was used to inoculate the flasks. They were then incubated and the spent medium treated as discussed in Section 5.2.1.1), to evaluate antimicrobial production activity. The results (data not shown) showed that using cross-species interaction did not appear to enhance the production of inhibitory molecules.

In conclusion, the shake flask cultures and different growth conditions employed in this stage of the study in an attempt to produce higher levels of antimicrobial compound(s) were shown to have no enhancing effects on the production of the inhibitory molecules.

5.2.2 Effect of physiochemical factors on the inhibitory molecule(s)

The presence of the antimicrobial compound in solution enabled a variety of experiments designed to probe its composition and physical properties. These included testing for the effects of physiochemical factors such as thermo-sensitivity, the antimicrobial molecules' stability at 9^{o}C, pH changes and enzyme tests were studied with the aim of discovering an interesting properties for this inhibitory compound, which may support our hypothesis that the marine isolate P, is potentially a new strain or at least is a strain that produces different antimicrobial compound(s). The SM of the antimicrobial producer *S. plymuthica* (P) was prepared as described in Section 5.2.1.1. The spent culture medium (SM) was initially concentrated using a centrifugal vacuum concentrator (Savant AES 2010 Speed Vac® System). Briefly, 20 ml of the SM was distributed by pipette, into a series of sterile 1.5ml microcentrifuge tubes. All the tubes were then placed into the vacuum concentrator and run for 3hrs at RT. The dried material in each tube was then re-suspended in 50µl of sterile distilled water, termed as concentrated spent media (CSM) samples, and stored with the SM in the refrigerator (9 ^{o}C), until used for assessing the influence of physiochemical factors (pH, enzymes and heat treatment), on antimicrobial activity as described below.

5.2.2.1 Effect of temperature on antimicrobial activity

I initially asked how does the inhibitory molecule(s) produced by strain P resist heat treatment? A series of 200µl aliquots of CSM in sterile 0.5ml microcentrifuge tubes were incubated at different temperatures (20, 50, 70 and 100 ^{o}C) for 15, 30, 60 and 120 minutes, respectively, in a dri-block heater. The tolerance of the antimicrobial compound to autoclaving was tested at 121 ^{o}C for 15 minutes, in an autoclave. The activity for the heat-treated CSM was tested by spot testing on NA plates, using MRSA, *S. epidermidis*, *Bacillus cereus var mycoides* (F), *Cl. difficile* strain 630 and *L. monocytogenes* Scott A as sensitive strains (Material and Methods, Section 2.1.5). The control used was CSM without any heat treatment.

5.2.2.2. Effect of proteinase K, amylase and lysozyme on antimicrobial activity

Studying such enzymatic effects on antimicrobial activity could provide us with preliminary characteristics with which to assess the nature of the antimicrobial compound(s). This was done by preparing solutions consisting of a combination of 95% (v/v) of CSM and 5% (w/v) of one of the following enzymes. Three different enzymes were used (proteinase K, β-amylase and lysozyme) and the samples were all tested and prepared as follows: (1) (70% proteinase K; 70µl of a 25 mg/ml proteinase K stock solution plus 30µl of distilled water and 95µl of the CSM). (2) (70% β-amylase; 70µl of a 25mg/ml β-amylase stock solution plus 30µl of distilled water and 95µl of the CSM). (3) (70% lysozyme; 70µl of a 25mg/ml lysozyme stock solution plus 30µl of distilled water and 95µl of the CSM). Afterwards, the tested samples were incubated at 37 ^{o}C for 5hrs. Subsequently, the antimicrobial activity was evaluated using the spot test as before. The controls were CSM without any treatment along with untreated enzyme solutions.

5.2.2.3 Effect of pH on antimicrobial activity

A further assay was performed to investigate how inhibitory molecule(s) was affected by pH changes. A series of 50 ml aliquots of SM in sterile 100 ml flasks were adjusted to pH

2, 4, 6, 8, 10, 12 and 14 using 1M NaOH or 1M HCl. Afterwards, the SM was concentrated and inhibitory activity was evaluated using a spot test as described previously.

The results of the effect of physiochemical factors on the inhibitory molecule(s) produced by strain P, revealed that the inhibitory molecule(s) was not affected by pH (pH 2 to 14) or by temperatures of up to 121 ^{o}C (data not shown). Furthermore, proteinase K, β-amylase and lysozyme did not affect the antimicrobial activity of the secreted inhibitory molecule(s) (Figure 5.4). The results of these physiochemical tests make it unlikely that the secreted inhibitory molecule is a protein.

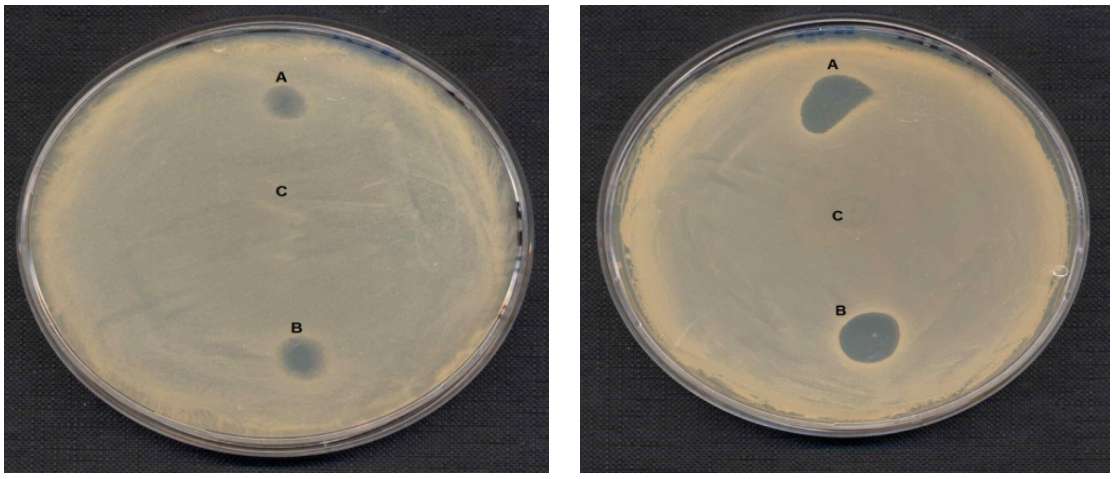


Figure 5.4 The effect of proteinase K on antimicrobial activity. Zones of inhibition on nutrient agar plates with test micro-organisms (*B. cereus var mycoides* (F) left panel and MRSA right panel). 20µl of concentrated spent media which had been exposed to 25 mg/ ml proteinase K for 5 hrs at 37 ^{o}C (A). (B) concentrated spent media (CSM) and (C) sterile distilled water (Negative control).

5.2.2.4 Effect of storage at 9^{o}C on the antimicrobial molecules' stability

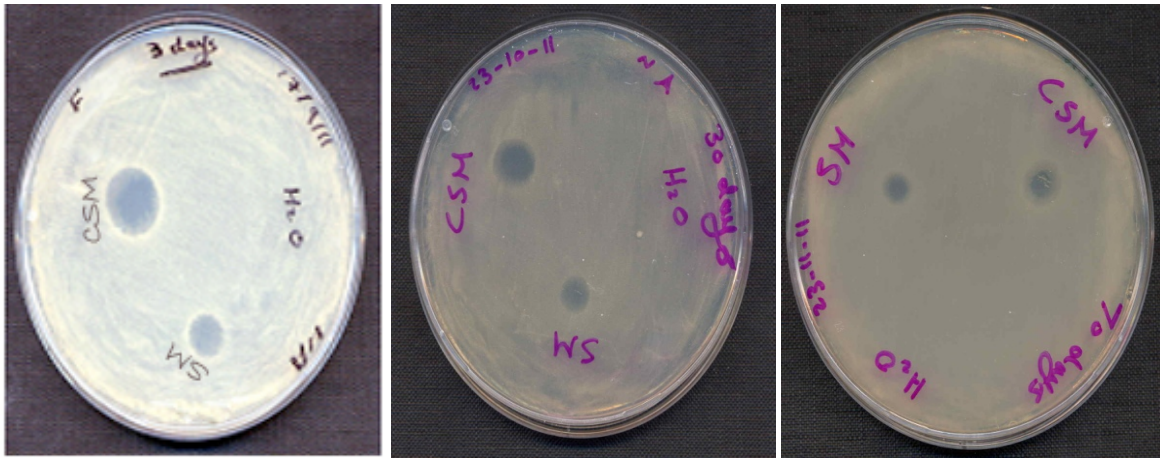
The effect of storage at 9 ^{o}C on the stability of unknown inhibitory molecule(s), is an important factor, in that the more stable it is the easier it will be to work with. Therefore, the stability and inhibitory activity of the bioactive molecule in spent media (SM) was assessed at various points during storage at 9 ^{o}C (1, 3, 5, 10, 15, 20, 25, 30, 40, 70 and 120 days), using the spot test (Materials and Methods, Section 2.1.5) on NA plates against *Bacillus cereus var mycoides* (F) and MRSA. CSM was used as positive control and H_2O

as a negative control. The results showed that the inhibitory molecule was stable for 25 days under these storage conditions, thereafter, the activity decreased as the storage time increased (Table 5.1 and Figure 5.5). Thus, the results suggest that an inhibitory molecule produced by this bacterium is relatively stable.

Table 5.1 The effect of shelf-life on the inhibitory molecule(s)' stability in the spent media (SM) as measured by its antimicrobial effects on *Bacillus cereus var mycoides* (F).

No. days stored	**Presence of antimicrobial activity**
1	+++
3	+++
5	+++
10	+++
15	+++
20	+++
25	+++
30	++
40	++
70	++
120	+

(+++) Very strong antimicrobial activity, (++) strong antimicrobial activity, (+) partial antimicrobial activity



A) B) C)

Figure 5.5 The effect of shelf-life on the antimicrobial molecule stability. A 10µl spot of spent growth media (SM) and concentrated spent growth media (CSM, positive control) and sterile distilled water (H_2O, negative control), was spotted onto a lawn of *Bacillus cereus var mycoides* (F) on NA plates incubated at 21 ^oC for 24hrs; A. 3 day storage; B. 30 day storage; C, 70 day storage.

5.2.3 Preliminary characterisation and purification of inhibitory molecule(s)

In order to gain an insight into the nature of antimicrobial compounds produced by strain (P), the purification of such compounds is essential. However, as biological samples usually contain mixtures comprised of several components, the use of size exclusion chromatography (SEC/ Gel filtration) and ion exchange chromatography (IEC), or a combination of several techniques based on different purification principles is needed for isolation, identification and characterization of inhibitory molecules.

5.2.3.1 Ultra filtration of the spent culture medium

As an initial step towards to identifying the inhibitory molecule (s) produced by *S. plymuthica* (P), I decided to use an ultra filtration technique in an attempt to give preliminary information about the molecular size of the antimicrobial compound. Molecular weight cut-off membrane filtration can be used to separate dissolved compounds in aqueous solutions on the basis of size: basically, small molecules will pass through the membrane pores while larger molecules will be retained at the membrane surface. The spent culture medium (SM) of the antimicrobial producer *S. plymuthica* (P),

was prepared as mentioned in Section 5.2.1.1. Then, it was passed through several molecular weight cut-off membranes (with the following molecular weight cut-off limits; 100,000, 50,000, 30,000, 10,000 and 5000 Daltons) (Materials and Methods Section 2.3.1). Antimicrobial activity of the spent media after passing through these membranes was then tested using the spot test method (Materials and Methods, Section 2.1.5) against the test micro-organisms (*Bacillus cereus var mycoides* (F)*, S. epidermidis*, *Clostridium difficile* strain 630 and MRSA. The ultra filtration of the SM appeared to show that the inhibitory molecule size is greater than, or equal to 5,000 Daltons (Table 5.2 and Figure 5.6).

Table 5.2 Preliminary sizing of the antimicrobial molecule in spent medium by ultrafiltration.

Membrane size molecular weight cut off (MWCO) in Daltons	**The presence of antimicrobial activity**			
	MRSA	*S. epidermidis*	*B. cereus*	*Cl. difficile*
<5,000	-	-	-	-
≥5,000	+++	+++	+++	+++
≥10,000	+++	+++	+++	+++
≥30,000	+++	+++	+++	+++
≥50,000	+++	+++	+++	+++
≥100,000	+++	+++	+++	+++
+Ve control (SM)	+++	+++	+++	+++
-Ve control (H_2O)	-	-	-	-

(+++) Very strong antimicrobial activity, (-) no antimicrobial activity, and spent culture medium (SM, +Ve control) and sterile distilled water (-Ve control). N= 3 times.

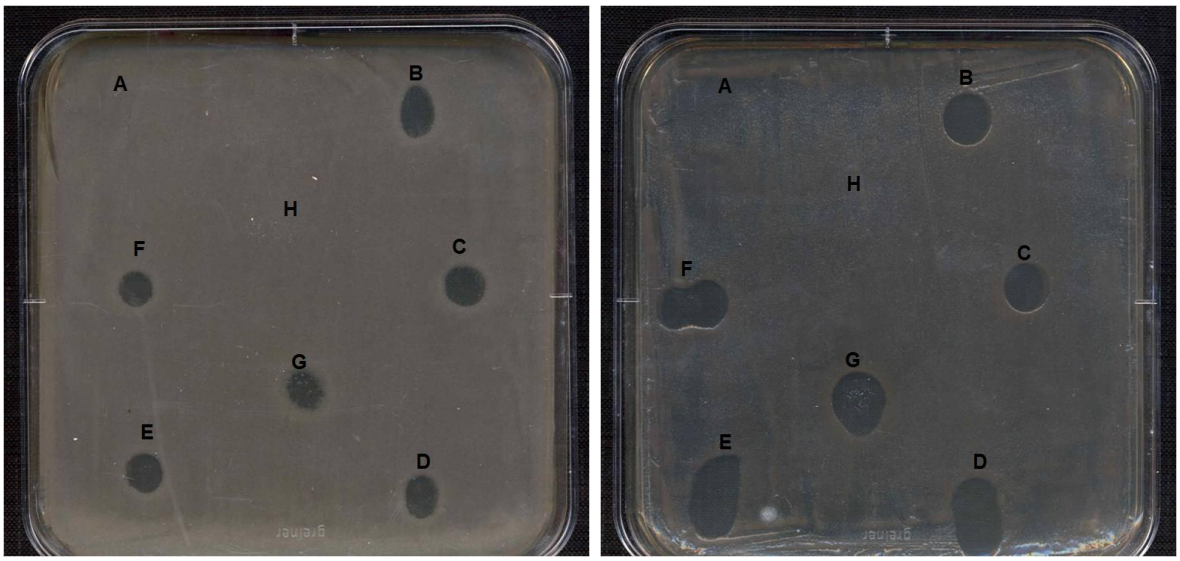
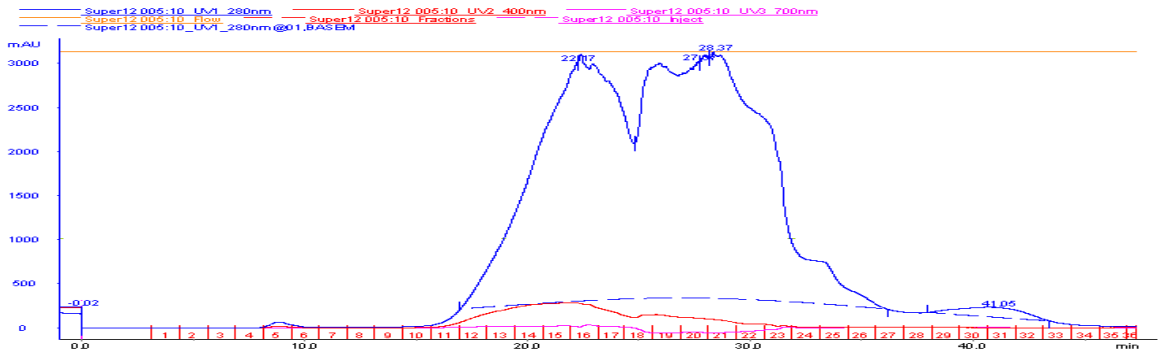


Figure 5.6 Zones of growth inhibition produced by spent medium after ultrafiltration.
20µl of the spent media that had passed through the various molecular weight cut-off membranes was spotted on to lawns of the test organisms (Left panel *B. cereus var mycoides* (F) and MRSA right panel) and the NA plates incubated overnight at 21°C. A (<5000 Da), B (≥ 5000 Da), C (≥10, 000 Da), D (≥30, 000 Da), E (≥50, 000 Da), F (≥100, 000Da), G (+Ve control, spent culture medium (SM)) and H (Sterile distilled water (-Ve control)). N= 3 times.

5.2.3.2 Purification of the antimicrobial molecule by size exclusion chromatography (SEC)

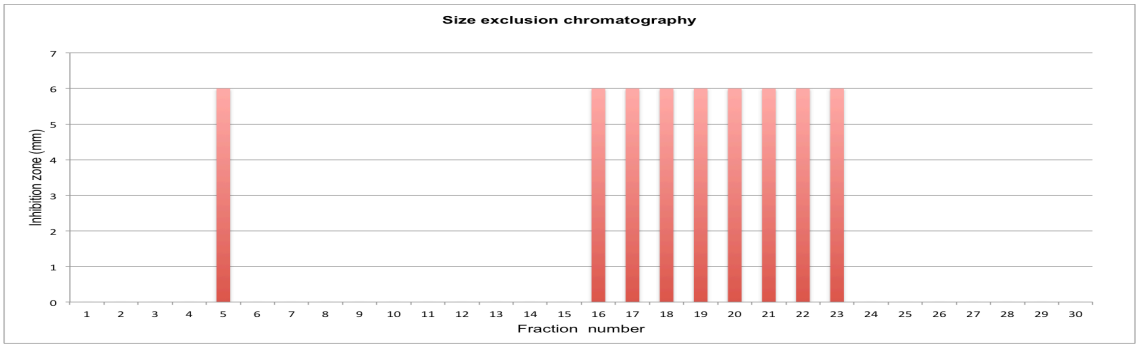
The molecular weight cut-off membrane experiments gave a relatively rapid and rough estimation of the size of the antimicrobial molecule. In order to provide more reliable information about the molecular size of the active compounds, the concentrated spent culture media (CSM, Section 5.2.2) from the antimicrobial producer was subjected to gel filtration (GF). 1ml of CSM was resuspended in the following buffer; 50mM Tris (pH7.5), 150 mM NaCl and 1mM EDTA and was injected onto a Superose®-12 column and run at a flow rate of 0.8ml/min. The fractions were monitored with UV light, at 280nm, and collected in 1.5ml microcentrifuge tubes. Fractions were then tested for antimicrobial activity test using a spot test (Materials and Methods, Section 2.1.5) against *Bacillus cereus var mycoides* on NA plates. The results shown in Figure 5.7; a, b and c show that nine fractions present in the HPLC analysis showed inhibitory activity against the indicator strain *B. cereus var mycoides* (F). Eight of the fractions (16, 17, 18, 19, 20, 21, 22 and 23), suggest an approximate molecular weight of ~1,000 Daltons while the remaining fraction (fraction No.5) appears to be a significantly higher molecular weight

compound (~2,000000 Daltons). These results suggest that the inhibitory compound(s) has a molecular weight of ~1,000 Daltons with evidence that the molecule may, under certain conditions, aggregate to produce a higher molecular weight complex. It is off course still possible that the high molecular weight peak represents a different compound. In contrast, this result is not in agreement with results obtained by molecular weight cut-off membranes (Section 5.2.3.1) that showed that bioactive molecules size is greater than, or equal to 5000 Daltons (Table 5.2 and Figure 5.6). An explanation for this could be that lots of larger molecules (≥5, 000 dalton) were retained at the surface of the cut-off membrane (≥ 5000 Da), consequently, blocking membrane pores and preventing small molecules to pass through membrane pores. However, the ultra filtration method alone cannot be used to characterize the active compounds.

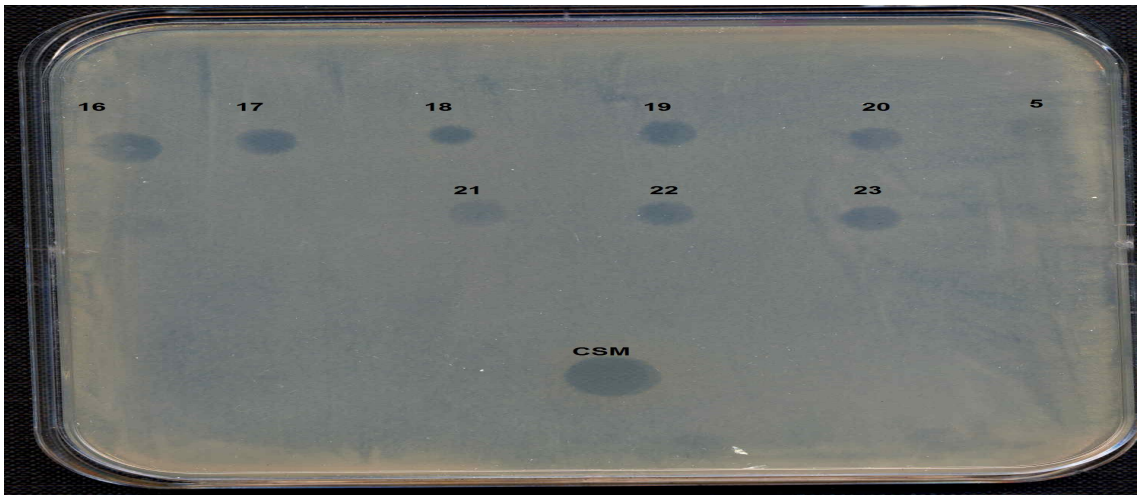


Sample Fractions

(a) A_{280nm} profile of the concentrated spent culture media (CSM)(blue line).



(b) Inhibition profile of gel filtration fractions 1-30.

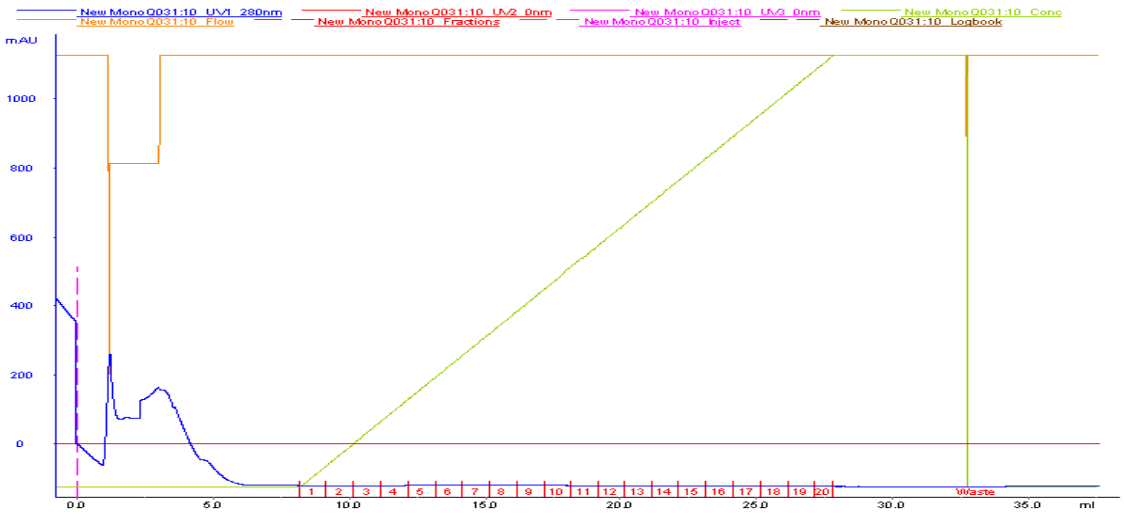


(c) Zones of clearing (10µl Spot test) resulting from the gel filtration fractions no (5, 16, 17, 18, 19, 20, 21, 22 and 23), on agar plates with confluent growth of *B. cereus var mycoides* (21 ^{o}C for 24hrs).

Figure 5.7 Gel filtration (GF). (a) A_{280nm} profile of the concentrated spent culture media (CSM) subjected to gel filtration. (b) Inhibition profile of gel filtration fractions 1-30. (C) Zones of clearing produced by the active antimicrobial fractions.

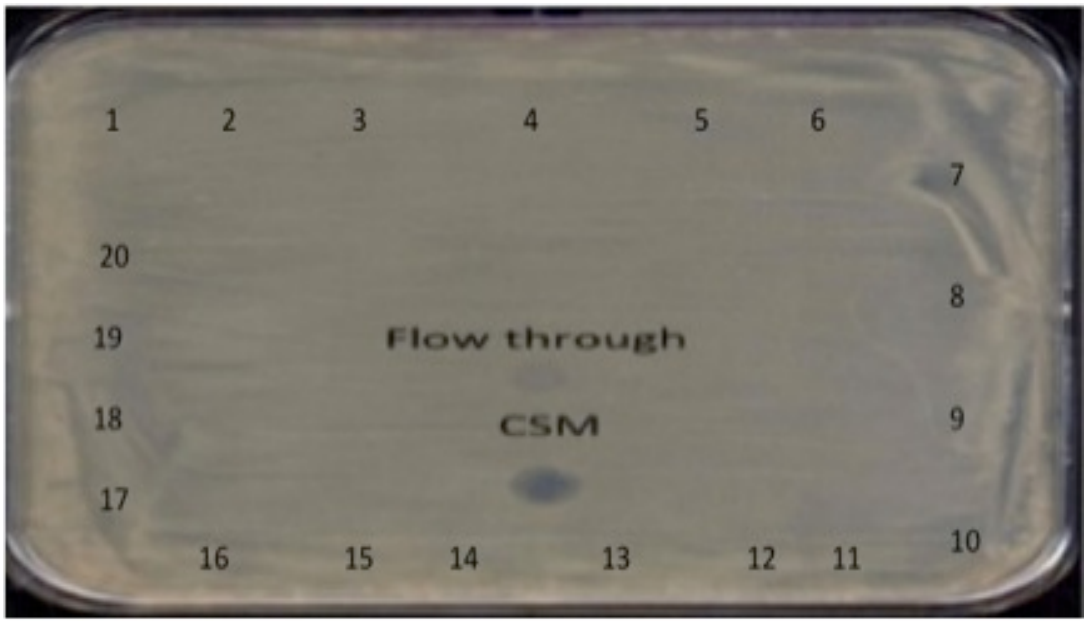
5.2.3.3 Anion exchange chromatography (AEC)

Further purification was conducted to gain an insight into the nature of the antimicrobial compound(s) produced by strain (P). Anion Exchange Chromatography (AEC) was used in this study to purify the inhibitory molecule(s), depending on their electrical charge (negative / positive) using a specific Anion exchange column. The bioactive fractions from the gel-filtration experiment above (5.2.3.2) were pooled (9ml in total), and concentrated as described in Section 2.3.2 (Materials and Methods), and then 1ml was subjected to the AEC, using a Mono-Q column the initial column running buffer was 50mM Na acetate pH5 plus 100mM NaCl and followed by a high salt elution buffer (50mM Na acetate pH5 plus 1M NaCl). The fractions eluting from the column were then tested for antimicrobial activity using the spot test (Materials and Methods, Section 2.1.5) against *Bacillus cereus var mycoides* on NA plates. The results showed that no inhibitory activity was found in the eluted fractions (1-20) (Figure 5.8; a and b) and only a small amount of activity was recovered in the column flow-through. This could mean that the inhibitory molecule(s) is either negatively charged or uncharged. Given the qualitative nature of the assay for the compound it is difficult to be sure that all the available bioactive material was recovered.



Sample Fractions

a) A280nm profile of anion exchange fractions (1-20).



b) Antimicrobial activity results (spot test) from anion exchange chromatography fractions (1-20), on agar plates with confluent growth of *B. cereus var mycoides*. Concentrated spent media (CSM) as a positive control. (plate incubated at 21 ^{o}C for 24hrs).

Figure 5.8 Anion Exchange Chromatography (AEC). (a) A_{280nm} profile of anion exchange fractions (1-20) (b) Antimicrobial activity results from anion exchange fractions (1-20), on agar plates with confluent growth of *B. cereus var mycoides.* The results showed that no inhibitory activity was found in the eluted fractions (1-20).

5.2.4 SDS-PAGE analysis

Based on the inhibitory molecule size data, obtained from ultra filtration and size exclusion chromatography (Gel filtration), it was determined that it would be beneficial to attempt to discover whether any proteins were present and to estimate their molecular weight. The inhibitory molecule within the spent media (SM), concentrated spent media (CSM) and the pooled active fractions from the gel filtration experiment were analysed using SDS-PAGE electrophoresis. The gel was run as described in Section 2.3.5 (Materials and Methods). The results shown in Figure 5.9 show that there was a low molecular weight band in lanes 4 and 5 (SM and CSM, respectively); however, the estimated size of the molecule will taken near the bottom of the gel, may be at too low a concentration to detect, and may not bind to Coomassie blue. No protein band was present at detectable levels in the pooled gel filtration fractions (lane 6).

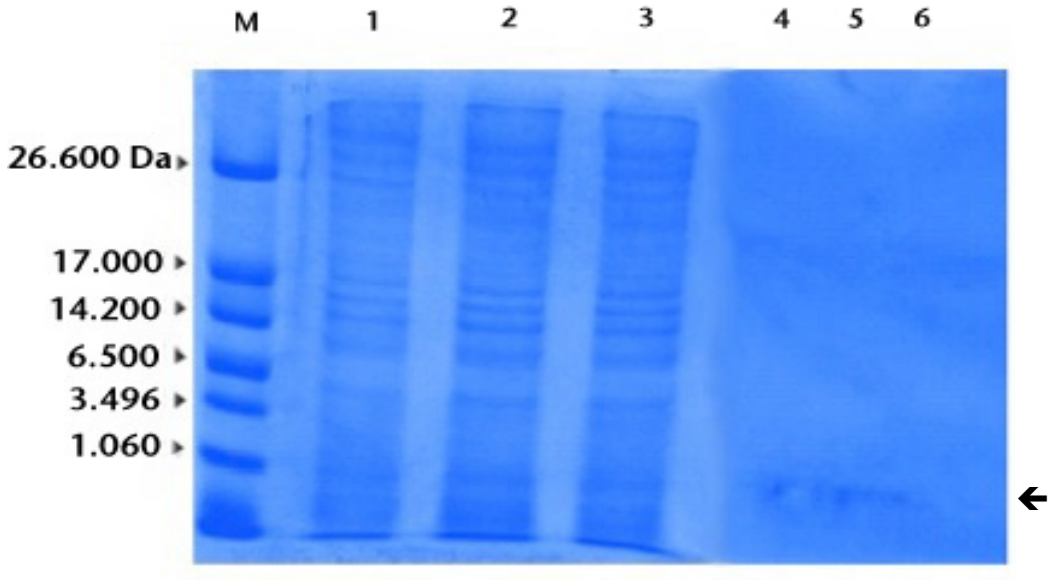


Figure 5.9 SDS-PAGE gel analysis. Lane M; the molecular mass marker, Lane 1, 2 and 3; crude extract protein of the marine isolate P, Lane 4; the spent media sample (SM), lane 5; concentrated spent media sample (CSM), and lane 6; Pooled bioactive fractions (fraction Nos. 5, 16, 17, 18, 19, 20, 21, 22 and 23) from the gel filtration experiment.

5.2.5 Is the inhibitory molecule bacteriostatic or bactericidal?

To establish whether the antimicrobial compound is either bacteriostatic or bactericidal, further experiments were carried out using the concentrated spent media (CSM, Section 5.2.2). The assay was done using a viable count plating method (Materials and Methods, Section 2.1.8), after the exposure of MRSA (as the sensitive strain) to different concentrations of the CSM. The tested samples were prepared as follows; a single colony of MRSA was inoculated into 10 ml of NB, and aerobically incubated with shaking (overnight at 37ºC). Following the period of incubation, 100µl of the overnight culture was transferred into 10ml of NB media and incubated at 37 ^{o}C for 5 hours (OD600nm= 0.7, late exponential phase). The cell culture was then divided into ten sterile 1.5ml microcentrifuge tubes, and centrifuged (13000 r.p.m for 5 min at RT) to obtain cell pellets. Different concentrations of CSM (0, 5, 10, 20, 30, 50,70,100 and 200%) (v/v) were then added to the pellets. The tubes (9 microcentrifuge tubes including the control (0% CSM = cell pellets without adding CSM) were then incubated aerobically at 37 ^{o}C for 4-hrs and total viable cell count was carried out as described in Section 2.1.8 (Material and Methods). The numbers of colonies was counted, following the incubation period (37 ^{o}C for 18-24hrs) and the results were recorded as a survival curve (% CFU/ml relative to the control), against CSM concentrations. The results shown in Figure 5.10 illustrate that the number of viable cells is decreasing. Thus, the inhibitory compound is bactericidal.

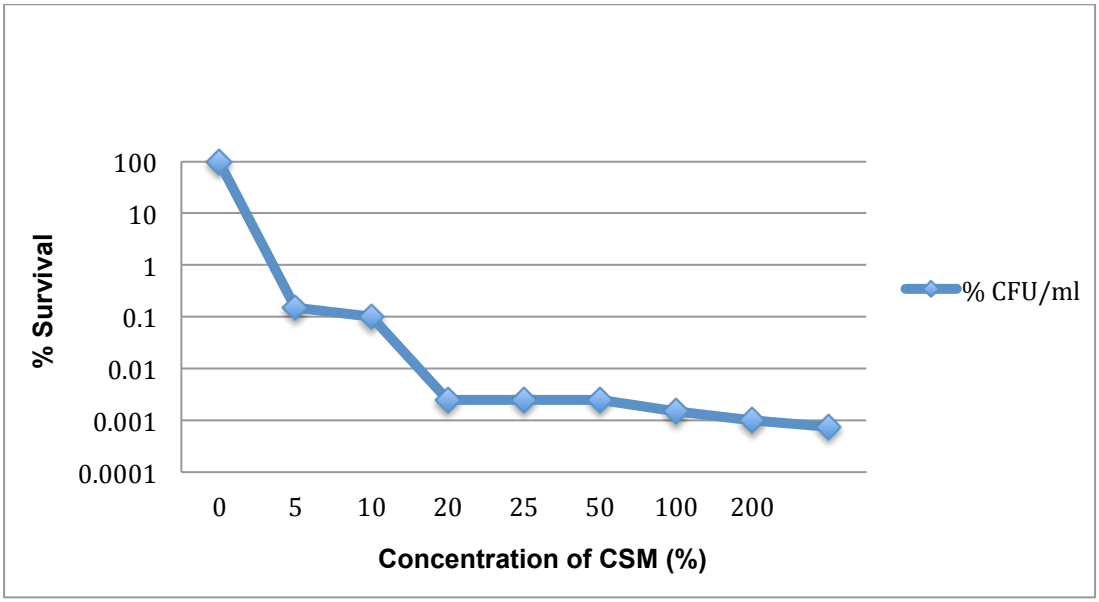


Figure 5.10 Cell survival of sensitive strain MRSA cells on exposure to increasing concentrations of CSM. Bacterial cultures were grown aerobically for 5 hours to a late exponential phase (OD600nm= 0.7) at 37 ^{o}C and cell pellets were treated with different concentrations of 0, 5, 10, 20, 30, 50, 70 100 and 200% (v/v) of CSM for 4 hours. 0% CSM = cell pellets without adding CSM. Total viable cell count was then carried out on NA plates and these were incubated overnight at 37 ^{o}C. The result represents a survival curve (% CFU/ml) against increasing concentrations of CSM. N= 2 times.

DISCUSSION

6. Discussion

Historically, the majority of current antimicrobials have been isolated from terrestrial organisms and only relatively recently have marine based organisms been the subject of research (Stach, 2010). The investigation of marine based micro-organisms has been largely successful, with researchers finding that they offer an abundant fresh source from which to collect new antimicrobials (Stach, 2010). This is significant because to date, roughly two-thirds of naturally occurring antibiotics have been isolated from bacterial sources, in particular from the genus actinomycetes. Many of these are of value for medical use in humans (Dharmaraj, 2010; Stach, 2010). It is therefore anticipated that by identifying new antimicrobials from marine micro-organisms it will be possible to find a new arsenal with which to overcome the increasing levels of resistance to current antibiotics.

Several of the currently available studies have focused on identifying novel antimicrobial compounds from specific marine bacterial species, primarily marine actinomycetes (Stach, 2010). However, so far research into other marine bacteria leading to the isolation of antimicrobials is limited; this is a motivation for further scientific research, as it is likely to be the study of rare marine micro-organisms that will lead to the emergence of new antimicrobial compounds. In recognition of the value of such research, this study has aimed to characterise antimicrobial producing micro-organism isolated from the surface of seaweed.

My results, as given in Chapters; 3, 4 and 5 have indicated that a micro-organism found on the surface of seaweed produces antibacterial metabolite(s) which react strongly against the Gram-positive foodborne pathogens: *Bacillus cereus var mycoides*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, multi-drug resistant human pathogenic bacteria *Clostridium difficile* strain 630, Vancomycin Resistant *Enterococci* (VRE), *Methicillin-resistant Staphylococcus aureus* (MRSA), and the foodborne spoilage organism *Penicillium expansum*. The work described utilised molecular microbiological techniques and classic microbiology as the basis for an initial characterisation of the marine bacterium and the inhibitory molecule(s). This work has extended our knowledge

about this natural antimicrobial producing bacterium, as well as providing further insights into its properties and potential applications in both industrial and clinical settings.

6.1 Screening for Antimicrobial Compound Production and Identification of the Antimicrobial Producing Marine Isolate (P)

When screening seaweed associated marine micro-organisms, for their ability to produce antimicrobial compounds, an organism was isolated as it was found to be producing antimicrobial metabolite(s); and was termed P. This marine micro-organism was screened for its ability to produce antimicrobial compounds and to kill or prevent the growth of a variety of different bacterial and fungal strains.

On the basis of the antimicrobial activity screening results (Results, chapter 3; Table 3.1 and Figure 3.1), the antimicrobial producing marine isolate, P, was shown to exhibit strong antimicrobial qualities against several micro-organisms, particularly those associated with foodborne spoilage such as *Penicillium expansum*, foodborne pathogens and multi-drug resistant human pathogenic bacteria such as (VRE) and (MRSA). Thus, this research has established that this strain produces a water-soluble inhibitory molecule(s). I would suggest that there is the presence of more than one molecule because of its activity against both Gram- positive bacteria, and fungal cells, due to the various differences between bacterial and eukaryotic micro-organisms, e.g., the differences between the cell wall structure. The antimicrobial activity screening results also showed that this strain (P) was particularly effective at inhibiting the growth of Gram-positive microorganisms, although it was inactive against Gram-negative pathogens. This finding is consistent with results from Austin *et al*, who also reported that antibiotics isolated from marine producing bacteria are typically more active against Gram-positive bacteria, with limited or no activity against Gram-negative bacteria (Austin, 1989). As the organism isolated in this study produced compound(s) that were active against pathogenic bacterial strains, including multi-drug resistant human bacterial pathogens (i.e. MRSA and VRE) and *Cl. difficile* strain 630, it is hypothesised that it may contain new antimicrobial agent(s).

In order to identify the marine micro-organisms (both P and F), a ribotyping analysis was applied. The 16S rRNA analysis results showed that the antimicrobial producer isolate, P,

has a high sequence identity (99%) to *Serratia plymuthica*, whereas the sensitive strain, F, showed high sequence identity (99%) to *Bacillus cereus var mycoides* (Results, chapter 3, Table 3.3 and Figure 3.8).

Strain P was further characterised by the use of phenotypic profiling and displayed the following phenotypic characteristics: it produced circular, convex, brick red pigmented, smooth surfaced round colonies, was Gram-negative, short rod shaped and non-spore forming (Results, chapter 3, Figure 3.2). This data corresponds with the characteristics that define the genus *Serratia* (Grimont and Grimont, 1978; Vivas *et al*., 2000). The organism was also positive for chitinase, proteinase, casinase, gelatinase lipase, DNase and haemolysis (Results, chapter 3, Figure 3.4). *Serratia* strains are generally known to produce extracellular components such as gelatinase, DNase and lipases (Farmer, 1985). The organisms was also salt tolerant which is in agreement with the characteristics defining the genus *Serratia* (they are generally salt tolerant) (Grimont and Grimont, 1978).

In general, *Serratia plymuthica* has received limited attention with respect to its antibacterial producing properties, in contrast to *S. marcescens*, (Stock *et al*., 2003; Harris *et al*., 2004). *S. plymuthica* strains have been shown to be potential pathogenic in humans and animals (Nieto *et al*., 1990). More recent studies have focussed on *S. plymuthica* strains isolated from soil with a potential application as bio-control agents against fungal pathogens (Compant *et al*., 2005; Meziane *et al*., 2006; Vleesschauwer and Hofte, 2007). To the best of my knowledge, this study is the first to attempt to investigate a marine isolate of *Serratia plymuthica*, in relation to its antibacterial properties.

To investigate the characteristics of the antimicrobial molecule(s) from marine *S. plymuthica* I asked: How similar, with respect to antimicrobial production, is strain (P) to the type strains of *S. plymuthica?* The similarity of this strain (*S. plymuthica* (P)) to other *S. plymuthica* strains (including the most common: *Serratia* spp, and the strain most frequently identified with human health: *S. marcescens*) was investigated. This investigation led to the conclusion that the marine strain is a new strain, as the spectrum of antimicrobial activity it generated was significantly different (Results, chapter 3, Table 3.4 and Figure 3.9). Further, the antimicrobials from strain *S. plymuthica* (P) are markedly different to those produced from *S. plymuthica* DSM 4540 and *S. marcescens* (ATCC13880). In this study, no antimicrobial activity was seen when using *S. plymuthica* DSM 4540 (reference strain) against strains that were sensitive to P. Crucially, I observed

that strain (P) resulted in an antimicrobial clearing zone against the *Bacillus cereus var mycoides* test strain (F) that was more uniform than that obtained when using *S. marcescens* (ATCC13880), although both inhibited the growth of this organism (Table 3.4 and Figure 3.9). Therefore, I can conclude that the marine environment isolated *S. plymuthica* (P) can be classified as a new strain that produces antimicrobial compound(s) that are different from similar strains derived from the terrestrial environment.

To obtain more detailed information about the properties of the antimicrobial producer strain (P), I carried out further investigations into the growth kinetics of the strain with regards to extrinsic factors. The tests performed to identify P are discussed below:

Growth conditions (media and temperature) were found to have some effect on the production of antimicrobial activity. The results showed that there was a decrease in the antimicrobial activity of the spent culture medium (SM) from cells grown in LB and MB media in comparison to the SM from TSB, BHI and NB grown cultures (Results, Chapter 5, Figure 5.1). This result is in agreement with the results obtained when evaluating the inhibitory activity of the live cultures of strain P that were grown on different agar media, which showed that the inhibition zone around the culture drop was much larger on the nutrient agar plates when compared with the other media tested (LB and TSA and BHI) (Results, Chapter 3, Figure 3.5). A possible explanation for this could be that the higher concentration of salt in the LB and MB media may suppresses inhibitory activity. Several other studies with different bacteria have also found a demonstrable influence of the chemical composition of growth media on antimicrobial activity (Martin and Demain, 1980; Farmer, 1985; Spížek and Tichý, 1995; Marwick *et al*., 1999).

Exposure to external factors such as temperature can also affect the functions of internal pathways, by controlling the expression of the genes involved in the production of antimicrobial compounds (Moons *et al.*, 2006). To establish the effects of growth conditions on antibacterial production, the effect of various temperatures (9 ^{o}C, 21 ^{o}C, 30 ^{o}C, 37 ^{o}C and 50 ^{o}C) was examined. These results showed that antimicrobial production was better at lower growth temperatures (i.e. below 30^{o}C), while the growth of strain P was better at 30^{o}C or above. In general, the production of the red pigment prodigiosin defines the genus *Serratia* (Farmer, 1985). As prodigiosin has been shown to have some antibacterial activity it was also important to determine if there was any link between antibacterial activity and production of the red pigment prodigiosin in strain P. The red

pigmentation was more prominent when grown on BHI agar plates, compared with the NA, LB and TSA media, with LB and TSA plates resulting in an intermediate level of red pigment (Results, chapter 3, Figure 3.6). Despite this, from my work there appeared to be no obvious link between prodigiosin production and antimicrobial activity.

A variety of different culture methods were utilised in an attempt to enhance the production of the antimicrobial agents. The effect of surface attachment on antimicrobial production was also examined, with the aim of stimulating the physical condition of the marine bacterium when it was growing on the surface of the plant (seaweed). For instance, Yan *et al.* (2003), reported that using such culture conditions led to the induction of antibacterial production when isolated from the marine environment (Yan *et al.*, 2003). Moreover, following exposure to the sensitive strain, antibiotic production from some marine epiphytic bacteria has also been reported (Burgess *et al.*, 1999). Unfortunately the use of different kinds of liquid culture conditions failed to enhance antimicrobial production from the marine *S. plymuthica* in this study, as did co-culturing strain P with a sensitive microorganism.

Some recent studies have explored the quorum-sensing abilities of *S. plymuthica*. Reports include the assertion that N-acylhomoserine lactones (AHLs) perform the role of signalling molecules in *Serratia* species, to regulate gene expression. This also includes a role in producing antimicrobial compounds and exoenzymes, butanediol fermentation and forming biofilms (Labbate *et al,.* 2004; Van Houdt *et al*., 2007a; Van Houdt *et al*., 2007b; and Pang *et al*,. 2008). Liu et al. (2011) characterises two QS systems for *S. plymuthica* strain G3 and determined their AHL profiles. They also asserted that QS molecules play a role in the regulation of antifungal activity. My results suggest that my strain of *S. plymuthica* (P) did not produce diffusible AHL molecules (Results, chapter 3, Figure 3.10). Again this result supports the hypothesis that strain P has unique and novel qualities.

6.2 Isolation and preliminary characterisation of mutants unable to produce the antimicrobial(s)

I decided to employ a Tn*5* mutagenesis method to isolate mutants of strain P, which failed to produce the antibacterial compound (Materials and Methods, Section 2.2.14). In this study, I identified, mutants deficient in antibacterial activity and subsequently cloned the transposons plus flanking genomic DNA from four of these mutants (M4, 5, 7 and 8). During the study, 14 mutants were obtained, one of these mutants was non-pigmented (M4), whereas the other thirteen were pigmented (Results, chapter 4, Figures; 4.3 and 4.4). There is no explanation for the lack of pigment in the mutant without pigmentation; experiments to prove or disprove whether the non-pigmented phenotype was due to the presence of the Tn*5* insertion were not done. These mutants were all shown to be the result of single Tn*5* insertions into the genome of strain P using a combination of PCR and Southern blotting techniques (Results, chapter 4, Figures; 4.5 and 4.7). The transposon insertion mutants should define some or all of the gene(s) responsible for the antimicrobial activity, although no attempt was made to determine the number of complementation groups. However, given the variation in the sizes of the genomic DNA regions flanking each Tn*5* and my data, it seems likely that the fourteen mutants define a more than one gene (Results, chapter 4, Figure 4.7 and Table 4.1).

One of the key features of the transposon mutagenesis system used is the ease with which it is possible to clone the insertion mutant and the adjacent genomic DNA. Genomic DNA is first cleaved with a restriction endonuclease, which does not cleave within the transposon, and the fragments are ligated; circular molecules containing the transposon then replicate as a plasmid (termed a transposon junction plasmid, TJP), in *E. coli* strains carrying the λ *pir* gene. Insertion mutants; 4, 5, 7 and 8 respectively were cloned (Results, chapter 4, Figure 4.8). However, attempts to clone the transposon insertion mutants from the following mutants (6, 9, 10, 11, 12, 13, 14, 15, 16 and 19) were unsuccessful; the reason for this is unknown.

The genomic region containing the transposon insertions for non-antibacterial producing mutants (4, 5, 7 and 8) (TJP 4, 5, 7 and 8) were partially sequenced as described previously in the material and methods chapter (Chapter 2, Figure 2.7). The data obtained from the transposon junction plasmid DNA sequencing was then compared to that found

in the Gen Bank using the BLAST-N and X programmes. Comparison of the resulting sequences against other sequenced *S. plymuthica* strains, such as *S. plymuthica* AS9, and the genome for *S. marcescens* strains (BLASTN), revealed no homology at the DNA level. Also, none of the four-transposon junction plasmid sequences obtained (4, 5, 7 and 8) were found to be related to the delivery vector (Results, chapter 4, Figure 4.9), although comparison of the resulting sequences (BLASTX) revealed that the four mutants (4, 5, 7 and 8) had insertions into gene(s) similar to those encoding non-ribosomal peptide polyketide synthases (Results, chapter 4, Figure 4.10). The results also revealed that these mutants (4, 5, 7 and 8) had insertions into gene(s) similar to those present in the other *Serratia* spp, although the level of homology is low, they were approximately 31% identical at the amino acid level and not at all at the nucleic acid level (Figure 4.10).

Non Ribosomal Peptides (NRPs) and Polyketides (PKs) are two classes of complex natural compounds produced by micro-organisms. They are metabolites with pharmacological properties that are considered to be important; e.g. Vancomycin - a non-ribosomal peptide antibiotic; Erythromycin - a polyketide antibiotic; and Epothilone - a mixture of both a polyketide and a non-ribosomal peptide that acts as an antitumor agent (Khosla *et al*., 1999; Schwarzer *et al*., 2003; Minowa *et al*., 2007). In general genes encoding non-ribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs) have been reported for the genus *Serratia* spp. A study performed on *Serratia* sp. ATCC 39006 and *S. marcescens* ATCC 274 to characterise the prodigiosin gene cluster (*pig* cluster), showed that several *pig* cluster gene products were similar to NRPSs, polyketide synthases and the red proteins of *Streptomyces coelicolor* (Harris *et al*., 2004). In addition, several studies have reported that (NRPs) and (PKs) can be produced by terrestrial isolates of *S. plymuthica*. For example, the first report of polyketide compounds produced by *Serratia plymuthica* strains was in 2001 by Thaning *et al.* who isolated a strain of *S. plymuthica* from soil. This strain produced a bioactive compound that inhibited the fungal plant pathogen *Sclerotinia sclerotiorum*. The compound was identified as a chlorinated macrolide and termed haterumalide NA. Subsequent to this, in 2004, Levenfors *et al.* isolated several inhibitory compounds from another soil derived *S. plymuthica* (A 153) including polyketide compounds (chlorinated macrolides and haterumalide (NA, B, NE and X). Another polyketide compound with antifungal activity was later identified by Shen *et al.* (2007); this had a chemical structure belonging to the chlorinated macrolide family and was termed macrocyclic lactone A21-4. In this study as there was essentially no homology between the transposon junction plasmid genomic

DNA sequences and related *Serratia* spp. at the nucleotide level (Results, chapter 4, Figures; 4.9 and 4.10), this supports my hypothesis that the marine isolate of *S. plymuthica* (P) is different to the other characterised strains.

Using the partial sequence data (Appendix 1), primers were designed in an attempt to clone the wild type genomic DNA for the antimicrobial gene(s), using long-PCR and cloning into pBluescript. I was able to amplify the wild type genomic DNA region corresponding to mutants 5, 7 and 8 but unfortunately I was unable to clone these fragments, even though the experiment was repeated carefully several times. The reason, why I could not get these constructs is unknown. For instance, it may be that there's been some form of recombination event between the insert DNA and the *E.coli* genome, probably unlikely as there is not significant homology between the sequenced portion of the inserts and *E. coli*. Or, it may be that the desired cloned gene was transcribed and translated, and the protein may then be toxic to the *E. coli*. In general, cloning genes involved antimicrobial production is usually more challenging than cloning many other types of genes (Martín and Gil, 1984).

In summary, the experiments have shown that Tn*5* mutagenesis was successful in disrupting and identifying at least some of the gene(s) associated with antimicrobial production in the marine *S. plymuthica* strain (P).

6.3 Preliminary characterisation of the antimicrobial agent(s)

To successfully characterise and gain insight into the antimicrobial compounds derived from strain P it is essential to determine its physical properties and to obtain a pure sample of the compound. Thermo-sensitivity, pH changes and the effect of enzymes on inhibitory compounds, are important features contributing to a preliminary understanding of the characteristics of the active molecule. While to obtain a pure compound several different purification steps are usually required, due to the many components present in biological samples.

The inhibitory activity produced by *S. plymuthica* (P) was found to be heat stable and the did not change after exposure to heat treatments of up to 121 ^{o}C. The inhibitory

compounds activity was also resistant to proteinase K (Results, chapter 5, Figure 5.1), β-amylase and lysozyme. In addition, the results also showed that altering the pH, from 2 to 14, did not affect the antibacterial activity. These results suggest that the secreted inhibitory molecule is unlikely to be a protein. This appears to contradict the sequencing results from my mutants, these suggested that the compound could be a non-ribosomaly encoded polypeptide (Results, chapter 4, Figure 4.10). However in my view, it is more likely that the proteins produced by these genes are responsible for the synthesis and modification of the antibacterial molecule, as opposed to being parts of the antibacterial compound, more work is required to determine if this is indeed the case.

The bioactive molecules produced by the isolated *S. plymuthica* strain in the spent culture media (SM) were found to be fairly stable at a storage temperatures of 9 ^{o}C for 25 days. This stability may be of interest and of significant value in both industrial and clinical applications. For instance, the more stable a compound is, the easier it will be to work with.

The effect of the compound on the survival and growth of MRSA was examined with a view to determining whether or not the compound was bactericidal or bacteriostatic. MRSA was used as the sensitive strain as the *B. cereus var mycoides* strain grows as filaments and consequently does not produce countable colonies. The results showed that the inhibitory compound is bactericidal (Results, Chapter 5, Figure 5.10). Following purification of the inhibitory molecules, further experiments should be performed to determine the minimum inhibitory concentration (MIC).

The spent culture media (SM), concentrated spent media (CSM) and the pooled active fractions from the gel filtration experiment, were analysed using SDS-PAGE electrophoresis to discover whether any proteins were present and to estimate their molecular weight. Figure 5.9 showed that there was a low molecular weight band in lanes 4 and 5 (SM and CSM, respectively); No protein bands were present at detectable levels in the pooled fractions. Indeed, a band on an SDS gel does not denote responsibility for antibacterial activity. However, any proteins present may be at too low a concentration to detect, and it seems very likely that the inhibitory compound may not bind to Coomassie blue.

As an initial step towards to identifying the inhibitory molecule(s) produced by *S. plymuthica* (P), I decided to use an ultra-filtration technique in an attempt to provide preliminary information about the molecular size of the antimicrobial compound. The data showed that the inhibitory compound was present in the SM and its size is greater than, or equal to 5,000 Daltons (Results, chapter 5, Table 5.2 and Figure 5.6). While this experiment gave a relatively rapid and rough estimation of the size of the antimicrobial molecule, to obtain better purification of the inhibitory molecule and confirm its molecular weight, a gel filtration fractionation was performed. This experiment split the antimicrobial activity into two distinct populations; one of a molecular weight of around ~1,000 Daltons (over eight fractions) and another much higher molecular weight fraction of ~2,000000 Daltons (Results, Chapter 5, Figure 5.7). All the previous studies have shown that the antibiotics produced by other strains of *S. plymuthica* are low molecular weight compounds. For instance, Shen *et al.*, reported that terrestrial isolates *S. plymuthica* A21-4 produce antibiotic macrocyclic lactone A21-4 of a low molecular weight, 470 Daltons (Shen *et al*,. 2007). In this study, it is not clear if my result represents two separate inhibitory molecules or whether this is evidence that under certain conditions the molecule is aggregating to produce a higher molecular weight complex. It is also possible that compound aggregation is behind the results obtained from the molecular weight cut-off membranes experiments. An alternative explanation would be that lots of larger molecules (≥5, 000 Daltons) were retained at the surface of the cut-off membrane (≥ 5000 Daltons), consequently, blocking the membrane pores and preventing smaller molecules from passing through the membrane pores. Therefore, I find that the ultra-filtration method alone is not reliable in characterising the active compounds. As at least one of the molecules appears to be small and relatively stable, as a result HPLC might be a more useful method for purification.

Anion Exchange Chromatography (AEC) was also used in this study to purify the inhibitory molecule(s); based on their electrical charge using a specific anion exchange column. The bioactive fractions from the GF assay was subjected to AEC using an anion exchanger column (Mono-Q, negatively charged). Unfortunately this was not successful, perhaps suggesting that either the inhibitory molecule(s) are negatively charged or uncharged.

In conclusion, I found that the active compound is heat resistant and has a low molecular mass ~1,000 Daltons and therefore is unlikely to be a protein. Most of the work

conducted on *S. plymuthica strains* is in the area of soil microbiology and biocontrol (Compant *et al*., 2005; Meziane *et al*., 2006; Matilla *et al*., 2012). However, as strain (P) was obtained from the marine environment and different strains of *Serratia* could be expected to yield multiple types of inhibitory molecules. This supports the notion that the marine environment isolate of *S. plymuthica* (P) is a new strain, one that produces novel antibacterial compound(s).

CONCLUSION

7. Conclusion

A microorganism, isolated from the surface of marine plants (seaweed; *Ascophyllum nodosum*), was found to produce antimicrobial metabolite(s) that were strongly active against several micro-organisms, particularly those micro-organisms associated with food borne spoilage, food borne pathogens and multi-resistant human pathogenic bacteria, such as VRE and MRSA. A combination of phenotyping and ribotyping of this marine bacterium indicated that it is very similar to *Serratia plymuthica*, a gram-negative micro-organism frequently associated with the surface of terrestrial plants. Our results showed significant phenotypic differences between the marine isolate and the other tested members of the *Serratia* genus of. The, preliminary characterisation of the inhibitory molecule(s) produced by this marine isolate demonstrated it was heat stable and of a low molecular weight (~1,000 Daltons), with the potential to be negatively charged or uncharged. It was found to be bactericidal towards at least MRSA. Preliminary identification and characterisation of the gene(s) involved antimicrobial production revealed that the four mutants (4, 5, 7 and 8) were inserted into gene(s) similar to those encoding non-ribosomal peptide/polyketide synthases.

To date, much of the work performed on *S. plymuthica* is within the field of soil microbiology and bio control. However, the present study, as far as I am aware, is unique in being the first study on a marine *S. plymuthica* isolated from the surface of seaweed. This study proposes that the marine *S. plymuthica* is a potentially a new strain, or at the very least that it is a strain that produces different antimicrobial compound(s). Consequently, it is an unexplored potential source of natural antimicrobial compound(s). Further investigations will be required to adequately characterise this marine bacterium. For example, it is still necessary to identify the all gene(s) responsible for the production of the antimicrobial agent(s) by cloning all the transposon insertions. Future research should aim to increase our knowledge of this natural antimicrobial producer bacterium, and to discover its properties and potential applications in an industrial setting.

APPENDICES

8. Appendices

8.1 Appendix (1)

8.1.1 Partial DNA sequence of mutant 5 (17-1direcion)

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|       10        20        30        40        50        60        70
CGATAGCTTAAGCATTTTGAGTGACACAGGACACTTAACGGCTGACATGGGGGGGTACCGAGCTCGAATT
GCTATCGAATTCGTAAAACTCACTGTGTCCTGTGAATTGCCGACTGTACCCCCCCATGGCTCGAGCTTAA

        80        90       100       110       120       130       140
CAGCGATGATGGTTGAGATGTGTATAAGAGACAGGGGTGGGTTTATTGATGATGCATATTGTTTCGATCC
GTCGCTACTACCAACTCTACACATATTCTCTGTCCCCACCCAAATAACTACTACGTATAACAAAGCTAGG

       150       160       170       180       190       200       210
CAAATTTTTTAGAATTTCCCCGCAAGAAGCTGAGTGGATGGATCCGCAAGCTCTGGTATTGTTGGAAGAG
GTTTAAAAAATCTTAAAGGGGCGTTCTTCGACTCACCTACCTAGGCGTTCGAGACCATAACAACCTTCTC

       220       230       240       250       260       270       280
AGCCTGAATGTTATCTACCATGCCGGTTATACTCATCATGAGCTTGCTGGTATGAATGTAGGTGTTTACA
TCGGACTTACAATAGATGGTACGGCCAATATGAGTAGTACTCGAACGACCATACTTACATCCACAAATGT

       290       300       310       320       330       340       350
TTGGAGCGCGTGGTCAACAAGTCAATCTTTCCAAAATCGAACATTGCCGTAATCCTATTATGGCGGTGGG
AACCTCGCGCACCAGTTGTTCAGTTAGAAAGGTTTTAGCTTGTAACGGCATTAGGATAATACCGCCACCC

       360       370       380       390       400       410       420
GCAAAACTATCTAGCCGCTAATATTTCGCAATTCTTCAATCTCAGAGGTCCCTCTTTGGTGATTGACACT
CGTTTTGATAGATCGGCGATTATAAAGCGTTAAGAAGTTAGAGTCTCCAGGGAGAAACCACTAACTGTGA

       430       440       450       460       470       480       490
GCCTGTTCGTCCTCTTTAGTTGGTATGAATATGGCAGTGGAAGCAATGTGCGCAGGTACTATTGACTCCG
CGGACAAGCAGGAGAAATCAACCATACTTATACCGTCACCTTCGTTACACGCGTCCATGATAACTGAGGC

       500       510       520       530       540       550       560
CGTTGGTTGGGGGGGTGAGTTTGCTGGCAAATTCGAGCGCCCATGAAATCTTTGCGCAACGTAATTTATT
GCAACCAACCCCCCCACTCAAACGACCGTTTAAGCTCGCGGGTACTTTAGAAACGCGTTGCATTAAATAA

       570       580       590       600       610       620       630
ACAAGCTAACGGAGAGTTTCATATCCTCGATCAGAGAGCATCTGGTGTCGTTCTTGGTGAAGGGTGTGGA
TGTTCGATTGCCTCTCAAAGTATAGGAGCTAGTCTCTCGTAGACCACAGCAAGAACCACTTCCCACACCT

       640       650       660       670       680       690       700
ATGGTTTATCTGAAGCCGCTTGAAAAGGCAAAGCAAGATGGTGATTGCATTTATGCTGTTATTGAAGGTA
TACCAAATAGACTTCGGCGAACTTTTCCGTTTCGTTCTACCACTAACGTAAATACGACAATAACTTCCAT

       710       720       730       740       750       760       770
TCGGAGTCAATAATGATGGCAGAACTGCAGGGCCCGCTACGCCTAACATGGCGGCTCAGGCCGATGTGAT
AGCCTCAGTTATTACTACCGTCTTGACGTCCCGGGCGATGCGGATTGTACCGCCGAGTCCGGCTACACTA

       780       790       800       810       820       830       840
GCAATCTGTGTTAAAGCAAAGCCAGTGTGCAAGCCATGACATAAGTTATCTGGATGTGAACGGATCTGGC
CGTTAGACACAATTTCGTTTCGGTCACACGTTCGGTACTGTATTCAATAGACCTACACTTGCCTAGACCG

       850       860       870       880       890       900       910
TCAGAAGTAACAGACTTGCTCGAGATAAAAGCAGTTGCGTCAGTCTACGCTTCAAAAGAAAGAAAGCCTC
AGTCTTCATTGTCTGAACGAGCTCTATTTTCGTCAACGCAGTCAGATGCGAAGTTTTCTTTCTTTCGGAG

       920       930       940       950       960       970       980
TGTATTTAGGATCTATGAAGCCAAATATTGGCCATCCTTTATGTGCAGAAGGTATTGCTAGCTTTATAAA
ACATAAATCCTAGATACTTCGGTTTATAACCGGTAGGAAATACACGTCTTCCATAACGATCGAAATATTT

       990      1000      1010      1020      1030      1040      1050
AGTTGCTTTGATGTTGCAGCACCAGCAGCTAGTTCCCTTTCTCTCTGGTCAAGAACCTCTTGCGCATTAT
TCAACGAAACTACAACGTCGTGGTCGTCGATCAAGGGAAAGAGAGACCAGTTCTTGGAGAACGCGTAATA
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…Sequence continued M5

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      1060       1070       1080       1090       1100       1110       1120
TCGATTGAGCGTTCGGGGTTTGCATTCCCAAGACAAGCACAAGCTTTTGAGATGGAATACGCTGCCTTGA
AGCTAACTCGCAAGCCCCAAACGTAAGGGTTCTGTTCGTGTTCGAAAACTCTACCTTATGCGACGGAACT

      1130       1140       1150       1160       1170       1180       1190
ATTGCTTCGCTGATGGTGGCACTAATGCTCATGTGATTTTGAGCAAATCATATCAAAACTAATTTTATCC
TAACGAAGCGACTACCACCGTGATTACGAGTACACTAAAACTCGTTTAGTATAGTTTTGATTAAAATAGG

      1200       1210       1220       1230       1240       1250       1260
TTTCACATTATCCTATTTAAATTTAGCTTCTTTCTCATTTTTGGAATATATATTCCGTATGGGGAAGAGG
AAAGTGTAATAGGATAAATTTAAATCGAAGAAAGAGTAAAAACCTTATATATAAGGCATACCCCTTCTCC

      1270      |1280       1290       1300       1310       1320       1330
ATTTTAATTAGCTTGATATATCATTTCATGAACTTAGCTAGCGGTATATCATTACTAGGCTTAAAGGAAT
TAAAATTAATCGAACTATATAGTAAAGTACTTGAATCGATCGCCATATAGTAATGATCCGAATTTCCTTA

      1340       1350       1360       1370       1380       1390       1400
ATGGACATGAATAATCTCAATTCAAATGCAAGAAAGAACAATGTTTCTGATGATCATTCAGTGAGCAAGC
TACCTGTACTTATTAGAGTTAAGTTTACGTTCTTTCTTGTTACAAAGACTACTAGTAAGTCACTCGTTCG

      1410       1420       1430       1440       1450       1460       1470
GGTCACCTCTGTCTCTGCCAACTATGCGTCGAGTGGATGTCCGGACACTTCAACTTACAGACAAGAAGAG
CCAGTGGAGACAGAGACGGTTGATACGCAGCTCACCTACAGGCCTGTGAAGTTGAATGTCTGTTCTTCTC

      1480       1490       1500       1510       1520       1530       1540
TGAAATTCATCAAGGAAGTGATAGCCTGGGTTATAGAACGCTAAGTCATTCATTTAAACCTTCAGGATGC
ACTTTAAGTAGTTCCTTCACTATCGGACCCAATATCTTGCGATTCAGTAAGTAAATTTGGAAGTCCTACG

      1550       1560       1570       1580       1590       1600       1610
TGGGATCTGATTCTCAGTAGCGAGCATCCGATCCTATCTGGTCACCAAGTGTATGGCCAGGAGTTATTGC
ACCCTAGACTAAGAGTCATCGCTCGTAGGCTAGGATAGACCAGTGGTTCACATACCGGTCCTCAATAACG

      1620       1630       1640       1650       1660       1670       1680
CAGGCCTTGCTTGGGTTGATTTCTTGTACCAGTGGTTCGCAGAGGCCGGATATTCATACGAGACGTTAGA
GTCCGGAACGAACCCAACTAAAGAACATGGTCACCAAGCGTCTCCGGCCTATAAGTATGCTCTGCAATCT

      1690       1700       1710       1720       1730       1740       1750
ATTAAAAAACTTATCCATTTATAGGCCTTTGATTATTTCAGAAAAAGAACAGGTAGCGTTATCGGTACAA
TAATTTTTTGAATAGGTAAATATCCGGAAACTAATAAAGTCTTTTTCTTGTCCATCGCAATAGCCATGTT

      1760       1770       1780       1790       1800       1810       1820
GCGATAGAGAGCAAAGAAGGATTTTGGACTGTTCAAGTTTCCAACGCTTCTATTGCCGCTACTGAGTCAG
CGCTATCTCTCGTTTCTTCCTAAAACCTGACAAGTTCAAAGGTTGCGAAGATAACGGCGATGACTCAGTC

      1830       1840       1850       1860       1870       1880       1890
CGCCATTGGCACTCTATATGTCGGCACAAATGCATCCGGTATCTGCGGTTTCATTTAAAGAGTCAGTTAA
GCGGTAACCGTGAGATATACAGCCGTGTTTACGTAGGCCATAGACGCCAAAGTAAATTTCTCAGTCAATT

      1900       1910       1920       1930       1940       1950       1960
TATTGACGAGGTTCTTGGCGGAAATGAGCCCCAGGTCGATTTCCTTCAACTTTATCAGAAATGCCAATCG
ATAACTGCTCCAAGAACCGCCTTTACTCGGGGTCCAGCTAAAGGAAGTTGAAATAGTCTTTACGGTTAGC

      1970       1980       1990       2000       2010       2020       2030
CGAGAGCTACTGCATACAGGAGTAATGAAAGCAAAAGGTGTGGTTTCTTCTAAAAATGGCACAACCTGGA
GCTCTCGATGACGTATGTCCTCATTACTTTCGTTTTCCACACCAAAGAAGATTTTTACCGTGTTGGACCT

      2040       2050       2060       2070       2080       2090       2100
TGCATGTTAAAGTGGATGAGGAGGTTCAAGTTGATACGCAAGACTTCTTGTTCCATCCCGCTCTGATTGA
ACGTACAATTTCACCTACTCCTCCAAGTTCAACTATGCGTTCTGAAGAACAAGGTAGGGCGAGACTAACT

      2110       2120       2130       2140       2150       2160       2170
CGGTAGTGGAGTGGGCGCTGGCACTTTACTGAGCAAGTTTGTGCCTGAAGAAACAAAGTTGTTTTACCCC
GCCATCACCTCACCCGCGACCGTGAAATGACTCGTTCAAACACGGACTTCTTTGTTTCAACAAAATGGGG

      2180       2190       2200       2210       2220       2230       2240
TATTNATCGAATCCTTNAATGCTCAGAGATGTTTGGTAGTGAATGCTATGTACGGATAAAAGAAGGGCTC
ATAANTAGCTTAGGAANTTACGAGTCTCTACAAACCATCACTTACGATACATGCCTATTTTCTTCCCGAG

      2250       2260       2270       2280       2290       2300       2310
ATAGAGATAAAAGAGGAGCTAATCAGTCTTACCATGGAGTTTTTAATTCTGAAGTAATAAAATGGGCGAG
TATCTCTATTTTCTCCTCGATTAGTCAGAATGGTACCTCAAAAATTAAGACTTCATTATTTTACCCGCTC


      2320       2330       2340       2350       2360       2370       2380
TAAGATTNCAAAATAATTGGTCGTCATGACGTTTAGTAAAAAGCAGAGCAAAAGTGCTCACTATGAAAGA
ATTCTAANGTTTTATTAACCAGCAGTACTGCAAATCATTTTTCGTCTCGTTTTCACGAGTGATACTTTCT
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…Sequence continued M5

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      2390       2400       2410       2420       2430       2440       2450
GATGATTAAAGAAAAGTCGTAGGGCCGGAACTACTTTGACCNGACTCCTATGCACCATTTACGCTATTGG
CTACTAATTTCTTTTCAGCATCCCGGCCTTGATGAAACTGGNCTGAGGATACGTGGTAAATGCGATAACC

      2460       2470       2480       2490       2500       2510       2520
ATGTGAACGAGATTGCTAAATGGTTTTGGATGGATGTTCCCCTTTTGAGCCAAAAATAACGGCCTCACTT
TACACTTGCTCTAACGATTTACCAAAACCTACCTACAAGGGGAAAACTCGGTTTTTATTGCCGGAGTGAA

      2530       2540       2550       2560       2570       2580       2590
CCCCCGGTTAAAAAAAAACTGGGACCGAAATTTTTTATTATCAACCAACGTTTTAAAAAATTTTTTTAAA
GGGGGCCAATTTTTTTTTGACCCTGGCTTTAAAAAATAATAGTTGGTTGCAAAATTTTTTAAAAAAATTT

      2600       2610       2620       2630       2640       2650       2660
AAAAAAAAAAAAAAAAAAGTGGTCTTTTTTTAGAAAAAGGGAANNNNNAAAAAAAAAAANANNNANNTTGTG
TTTTTTTTTTTTTTTTTCACCAGAAAAAAATCTTTTTCCCTTNNNNNTTTTTTTTTTNTNNNTNNAACAC

      2670
AGTGGGGTATAAAAAAA
TCACCCCATATTTTTTT
```

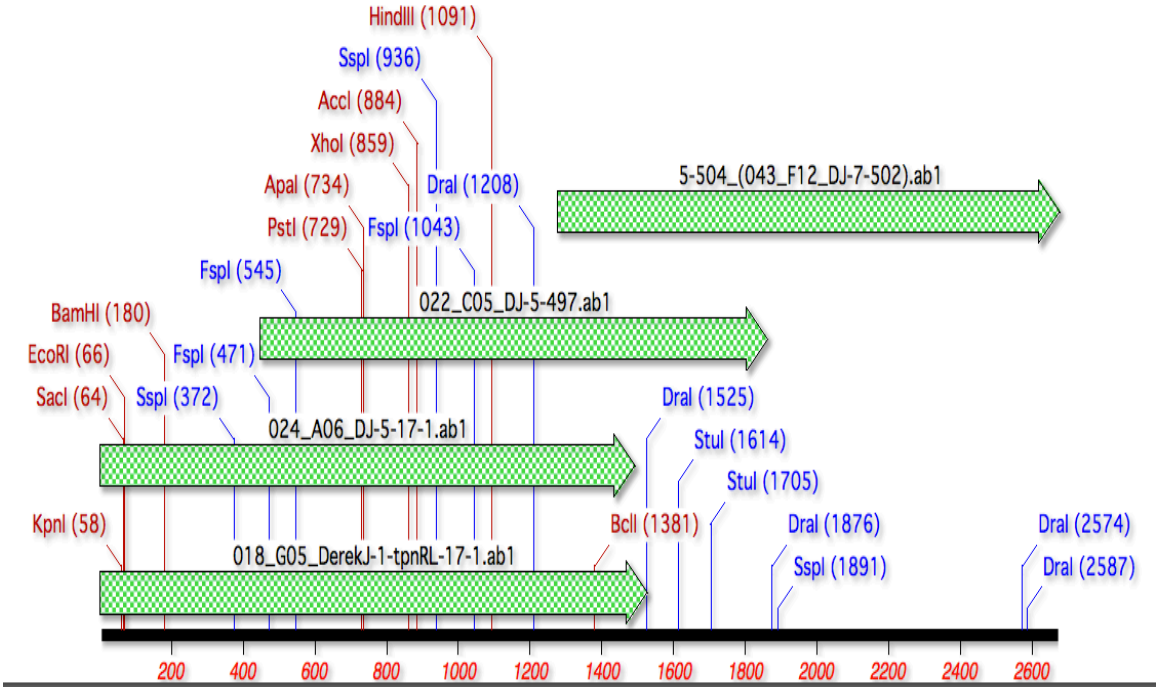

Figure 8.1 Graphic map of sequence contig obtained from Mutant No.5, primer tpnRL17–1 direction. *Bam*H1site identified on the base pair No. 180. Each green arrow represents the length of sequence coverage and the figure shows the level of sequence overlap between each sequence run.

8.1.2 Partial DNA sequence of mutant 7 (17-1direcion)

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|
|       10        20        30        40        50        60        70
CCACTAACCTAAGCATTTTGAGTGCACAGGAACACTTAACGGCTGACATGGGGGGGTACCGAGCTCGCAT
GGTGATTGGATTCGTAAAACTCACGTGTCCTTGTGAATTGCCGACTGTACCCCCCCATGGCTCGAGCGTA
        80        90       100       110       120       130       140
TTAAAGATGATGGTTGAGATGTGTATAAGAGACAGGCTTTCCATGACAGGCTGTCAAGCGAGAAGCTGAA
AATTTCTACTACCAACTCTACACATATTCTCTGTCCGAAAGGTACTGTCCGACAGTTCGCTCTTCGACTT
       150       160       170       180       190       200       210
CATGCCTCGCACGCATCGAATTTGCTAACAGTTGCAGAGAAATGGGTAAATGGAACAAGGTTCTCGTGGG
GTACGGAGCGTGCGTAGCTTAAACGATTGTCAACGTCTCTTTACCCATTTACCTTGTTCCAAGAGCACCC
       220       230       240       250       260       270       280
ATGATTTCAATAAAGGAAATGAGCAAAAAGTTCCTTTACCTACCTATCCTTTTGCAAAAGAGAGGCATTG
TACTAAAGTTATTTCCTTTACTCGTTTTTCAAGGAAATGGATGGATAGGAAAACGTTTTCTCTCCGTAAC
       290       300       310       320       330  |    340       350
GGTCAGGGCTGATGGCGTACCGGAAGCCTTTGACCAAAATGATCAAGTAGGTGCTAATGCAGCACTTGGA
CCAGTCCCGACTACCGCATGGCCTTCGGAAACTGGTTTTACTAGTTCATCCACGATTACGTCGTGAACCT
       360       370       380       390       400       410       420
AACGAACAAGATAGCTCTACTGAGGATACTTTACGCATAAAAGTAGAAGACTACCTTAAAGCTATTTTCT
TTGCTTGTTCTATCGAGATGACTCCTATGAAATGCGTATTTTCATCTTCTGATGGAATTTCGATAAAAGA
       430       440       450       460       470       480       490
CTGAAGTGTCTGAGATCGCCATTTCACGCATTGGCTCAGAAGCCACATTTGATGAGTATGGAATTAACTC
GACTTCACAGACTCTAGCGGTAAAGTGCGTAACCGAGTCTTCGGTGTAAACTACTCATACCTTAATTGAG
       500       510       520       530       540       550       560
GCTAATGATAACGAAACTTAACTCACTTCTTGAAAAAGATTTTGGCGAGTTATCAAAAACGTTATTTTTC
CGATTACTATTGCTTTGAATTGAGTGAAGAACTTTTTCTAAAACCGCTCAATAGTTTTTGCAATAAAAAG
       570       580       590       600       610       620       630
GAGTATCAATCTATCCGCGGGTTAGCTAATTACTTTTTAATCAACCATTTAGGAAAACTAAACACACGAT
CTCATAGTTAGATAGGCGCCCAATCGATTAATGAAAAATTAGTTGGTAAATCCTTTTGATTTGTGTGCTA
       640       650       660       670       680       690       700
TCAATCATACTGATTCATCAGCAGTTAAAAAGGCCAGTAGCAGTAATGACGTCATTAAAGAATCTGGAAG
AGTTAGTATGACTAAGTAGTCGTCAATTTTTCCGGTCATCGTCATTACTGCAGTAATTTCTTAGACCTTC
       710       720       730       740       750       760       770
GAAAGGCATTGTTTTATCCACTTCTGATTCGCAAACGGAAAAGGCATGTGATGTTGCTATTATTGGCTTA
CTTTCCGTAACAAAATAGGTGAAGACTAAGCGTTTGCCTTTTCCGTACACTACAACGATAATAACCGAAT
       780       790       800       810       820       830       840
AGTGGTAAGTACCCTCGGTCTGATACTGTTGAAGAGTATTGGGACAATCTACAGAATGCGGTTGATTGTA
TCACCATTCATGGGAGCCAGACTATGACAACTTCTCATAACCCTGTTAGATGTCTTACGCCAACTAACAT
       850       860       870       880       890       900       910
TTTCTGAAATACCTAAATCGAGATGGGATTATCACGATTATTATGATCCGGATAGAACTTCACAAGGAAC
AAAGACTTTATGGATTTAGCTCTACCCTAATAGTGCTAATAATACTAGGCCTATCTTGAAGTGTTCCTTG
       920       930       940       950       960       970       980
GATCCGTTCTAAATGGGGGGGGTTCATAAATGGTGTTGATGAATTTGATCCTCTGTTTTTCAATATATCT
CTAGGCAAGATTTACCCCCCCCAAGTATTTACCACAACTACTTAAACTAGGAGACAAAAAGTTATATAGA
       990      1000      1010      1020      1030      1040     |1050
CCTCGCGAAGCTGAGTTTATGGATCCTAAAACACCAAAGGAACGGCTCGAATTTATTTTGCAAGATGCAA
GGAGCGCTTCGACTCAAATACCTAGGATTTTGTGGTTTCCTTGCCGAGCTTAAATAAAACGTTCTACGTT
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…Sequence continued M7

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      1060       1070       1080       1090       1100       1110       1120
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TACATAGACACTAAAATGCGTCCCTTTACCCAAAGAAACCATCTACCAATAGTCCCTTCTCACTAACGAA
      1130       1140       1150       1160       1170       1180       1190
TAGATCGGAACAACTTTTCAGAGCAAAGAACAACAAATCTAACCAGTGTTAACCAGCCAGATGACTTGGC
ATCTAGCCTTGTTGAAAAGTCTCGTTTCTTGTTGTTTAGATTGGTCACAATTGGTCGGTCTACTGAACCG
      1200       1210       1220       1230       1240       1250       1260
GTACATAATCTATACTTCAGGTTCTACGGGTAAACCTAAGGGTTGTATGTTGCCTCATGAAGCTATTTGT
CATGTATTAGATATGAAGTCCAAGATGCCCATTTGGATTCCCAACATACAACGGAGTACTTCGATAAACA
      1270       1280       1290       1300       1310       1320       1330
AATCGCTTACTTTGGATGAAAGATCAGTATCAAGTGAGTAACAGTGACCGTATATTACAAAAGACCCCCT
TTAGCGAATGAAACCTACTTTCTAGTCATAGTTCACTCATTGTCACTGGCATATAATGTTTTCTGGGGGA
      1340       1350       1360       1370       1380       1390       1400
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TATGTAAGCTACACAGCCATACCCTCGACAAAGAAGGAAACAATAGACCACGAACGGAACAAAAACGATT
      1410       1420       1430       1440       1450       1460       1470
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TGGATGTCCCGTATCTCTATTACGTATAAATTAGTTGGAATAGGTTGTTCTCTTTCAGTGGTAAACAGTA
      1480       1490       1500       1510       1520       1530       1540
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AAACAAGGAAGTTACAACGCTAAGAAAAACTTGGTTTTACGCCGGAGTGTGACGTGCAGTAACTTCGTGC
      1550       1560       1570       1580       1590       1600       1610
TATTTGCTAGTGGTGAAGCGCTGCCTTATGAATTGGTTGACAAATTTATAAGTACGATGCCCGCTAAATT
ATAAACGATCACCACTTCGCGACGGAATACTTAACCAACTGTTTAAATATTCATGCTACGGGCGATTTAA
      1620       1630       1640       1650       1660       1670       1680
GCATAACCTATATGGCCCTACAGAAGCAGCTGTCGATGTAAGCTATTGGCAGTGTGAATTACGCCAAGAT
CGTATTGGATATACCGGGATGTCTTCGTCGACAGCTACATTCGATAACCGTCACACTTAATGCGGTTCTA
      1690       1700       1710       1720       1730       1740       1750
AAAAAGGTGCCTATTGGTCGGGAAATTAGCAACGTGCAGCTTCATGTACTGAATGAGCAGTTGGTTCCAG
TTTTTCCACGGATAACCAGCCCTTTAATCGTTGCACGTCGAAGTACATGACTTACTCGTCAACCAAGGTC
      1760       1770       1780       1790       1800       1810       1820
TCGAGCAGGGAGATAGTGGCGAACTGTATATCGGCGGAATTTGTTTGGCCAAAGGTTATTTAAATAGACC
AGCTCGTCCCTCTATCACCGCTTGACATATAGCCGCCTTAAACAAACCGGTTTCCAATAAATTTATCTGG
      1830       1840       1850       1860       1870       1880       1890
GGAACTCACGATAGAGCGATTTATTGATAATCCACTGGTTAGCGCTGCTGAAAAAAGTCAGAAATATATA
CCTTGAGTGCTATCTCGCTAAATAACTATTAGGTGACCAATCGCGACGACTTTTTTCAGTCTTTATATAT
      1900       1910       1920       1930       1940       1950       1960
GGACTGGTGATAAAGCGCGCTGCTTGTCTGACGGAATATTGAATATTAAGCCCGCCTGGACTCTCAGGTT
CCTGACCACTATTTCGCGCGACGAACAGACTGCCTTATAACTTATAATTCGGGCGGACCTGAGAGTCCAA
      1970       1980       1990       2000       2010       2020       2030
AATTACCAGGTTTCGCATAGATTAAGTGATTTGAAAGTAAGCTTAATAACTAAACTATTGATCCCTCGGG
TTAATGGTCCAAAGCGTATCTAATTCACTAAACTTTCATTCGAATTATTGATTTGATAACTAGGGAGCCC
      2040       2050       2060       2070       2080       2090       2100
TATTGGAAAGGAGAGGCTCGTTCACCCAGTTATTGCTTATGTGTACAAAACCGTATAATTTAAGAAGTAG
ATAACCTTTCCTCTCCGAGCAAGTGGGTCAATAACGAATACACATGTTTTGGCATATTAAATTCTTCATC
      2110       2120       2130       2140       2150       2160       2170
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TTAAAGAATTTGTCGGCGCCTATTAGACGTTTCACACCACGAATTCTTGGGCATTCTTTCTTTTTACGGT
      2180       2190       2200       2210       2220       2230       2240
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TCGAAAAAAAAAGGTTATTGTCTTTAGCCCCTTTAGGAGTTTTTGAATTGACTAAGTCTTATTAGCTTCCC
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…Sequence continued M7

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      2250       2260       2270       2280       2290       2300       2310
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TTTTTGCACGTAAACGTTACTACGAAGGGAATTGCTTCCTCCTTTTAAAGCCCTGGAAGACGCCACTTTG
      2320       2330       2340       2350       2360       2370       2380
AAATGTCGGGGGGTTATCCAAAATCTTTGGGTACCCACACATTCCCCTCCTGGGTGGCTTTTTTCCGTAT
TTTACAGCCCCCCAATAGGTTTTAGAAACCCATGGGTGTGTAAGGGGAGGACCCACCGAAAAAAGGCATA
      2390       2400       2410       2420
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GAAATGAGAACATGCTTATGTCAAACTTTGTGAATAAATTAA
```

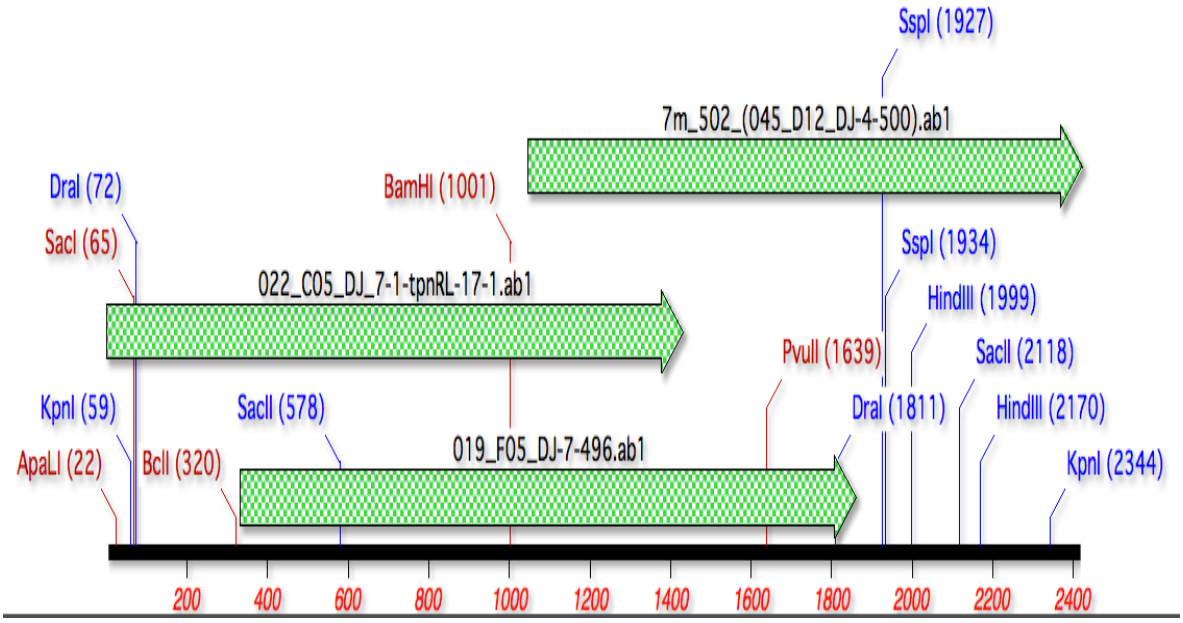

Figure 8.2 Graphic map of sequence contig obtained from Mutant No.7, primer tpnRL17–1 direction. *Bam*H1site identified on the base pair No. 1001. Each green arrow represent the length of sequence coverage and the figure show the level of sequence overlap between each sequence run.

8.1.3 Partial DNA sequence of mutant 8 (17-1direcion)

```
|
|        10         20         30         40         50         60         70
CGGCCAAACCTTAAGCCTCTCAAGCAATTTTGAGTGACACAGGAACACTTAACGGCTGACATGGTGGGGG
GCCGGTTTGGAATTCGGAGAGTTCGTTAAAACTCACTGTGTCCTTGTGAATTGCCGACTGTACCACCCCC
         80         90        100        110        120        130        140
TACCGAGCTCGAATTCATCGATGATGGTTGAGATGTGTATAAGAGACAGATCTCTGCCTGAGTAATTTTT
ATGGCTCGAGCTTAAGTAGCTACTACCAACTCTACACATATTCTCTGTCTAGAGACGGACTCATTAAAAA
        150        160        170        180        190        200        210
CATATACAAAGTCCCTTCGTTGATCTTTAAAGTCGCTAGATAAATTTTTTATAGGGCGCACTCAACCATG
GTATATGTTTCAGGGAAGCAACTAGAAATTTCAGCGATCTATTTAAAAAATATCCCGCGTGAGTTGGTAC
        220        230        240        250        260        270        280
AATGGCAGGTACAGTCTCTAATAACAGAATGAAATATTATTGAGCACGTAAACCGGATTAACACAACAAG
TTACCGTCCATGTCAGAGATTATTGTCTTACTTTATAATAACTCGTGCATTTGGCCTAATTGTGTTGTTC
        290        300        310        320        330        340        350
TGGAACACTTAGTCTAAATAAAACTTCACCCGCGCCAACTCATCCAGAGTCCGCCTAAAAATCATATTCA
ACCTTGTGAATCAGATTTATTTTGAAGTGGGCGCGGTTGAGTAGGTCTCAGGCGGATTTTTAGTATAAGT
        360        370        380        390        400        410        420
AGACAAATCCATATATCTTACTCTTCCAGACCATCTAACCAATCACCGAAACGAGTTCTGCCGTCAACGA
TCTGTTTAGGTATATAGAATGAGAAGGTCTGGTAGATTGGTTAGTGGCTTTGCTCAAGACGGCAGTTGCT
        430        440        450        460        470        480        490
TTTCAGGTCGCTCTGTCATTCCGACATAATGCTCATTACCGTCTTGCACTTACCCCTTTACCGCAGCGAG
AAAGTCCAGCGAGACAGTAAGGCTGTATTACGAGTAATGGCAGAACGTGAATGGGGAAATGGCGTCGCTC
        500        510        520        530        540        550        560
AAAGCTTTCTATATCACCTCCTTTATTGCTCCTATCACACACCTTGTAAACAAAGTGCTGTATTGACACC
TTTCGAAAGATATAGTGGAGGAAATAACGAGGATAGTGTGTGGAACATTTGTTTCACGACATAACTGTGG
        570        580        590        600        610        620        630
ACCAAAACCCATACTCATAGTTAATGCACTTTCAATTTTTAAAGATTCTGCTTGGTGCCTAACCCAATTA
TGGTTTTGGGTATGAGTATCAATTACGTGAAAGTTAAAAATTTCTAAGACGAACCACGGATTGGGTTAAT
        640        650        660        670        680        690        700
AAAGATGGCTCGATGGGGTTATCCAAATTTCTTGTTGGGTGTAATTTGTTCTCTTTCATTTGCAATAGAG
TTTCTACCGAGCTACCCCAATAGGTTTAAAGAACAACCCACATTAAACAAGAGAAAGTAAACGTTATCTC
        710        720        730        740        750        760        770
TTGCGACCACTTCTACCGTACCGGCTGCACTCAAGCCATGACCAATAATAGATTTAGTTGCATTAAGACG
AACGCTGGTGAAGATGGCATGGCCGACGTGAGTTCGGTACTGGTTATTATCTAAATCAACGTAATTCTGC
        780        790        800        810        820        830        840
AGCATGATCCAGTTCACAGTGTCGAAATGCTCGTAGTTCTGTCTCATCTCCAATACTAGAGCTTGTCCCA
TCGTACTAGGTCAAGTGTCACAGCTTTACGAGCATCAAGACAGAGTAGAGGTTATGATCTCGAACAGGGT
        850        860        870        880        890        900        910
TGGGGGATTGATGTAGTCAATCTCGCTTGCTGTTAAATTTGCGCGTTCTAAAGCATTTTTTATTGCTCTA
ACCCCCTAACTACATCAGTTAGAGCGAACGACAATTTAAACGCGCAAGATTTCGTAAAAAATAACGAGAT
        920        930        940        950        960        970        980
ACTTCGCCATCAAAAGAAGGGTTAGGATTCCTATTTCCATCCATTTCCACTGCCCAACCAGAAACGCGTG
TGAAGCGGTAGTTTTCTTCCCAATCCTAAGGATAAAGGTAGGTAAAGGTGACGGGTTGGTCTTTGCGCAC
        990       1000       1010       1020       1030       1040       1050
CATAGGGCTTCACTCTTTGCCTTAAATTAGCTCTTTCGATGACAACGACACCACAAGATTCCCCATATAT
GTATCCCGAAGTGAGAAACGGAATTTAATCGAGAAAGCTACTGTTGCTGTGGTGTTCTAAGGGGTATATA
       1060       1070       1080       1090       1100       1110       1120
GAATCCATCTCTCTCTTTGTCAAACGGACGACACGCCAATGCTGCTTCGTTTGCAAAACGATCTGACCCC
CTTAGGTAGAGAGAGAAACAGTTTGCCTGCTGTGCGGTTACGACGAAGCAAACGTTTTGCTAGACTGGGG
```

…Sequence continued M8

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      1130       1140       1150       1160       1170       1180       1190
ATAGCCCCCAAAGAACGGAATCCTTGACACTCCCAATATGACAAATCCATAAGTGCGCCGATAGCAATAC
TATCGGGGGTTTCTTGCCTTAGGAACTGTGAGGGTTATACTGTTTAGGTATTCACGCGGCTATCGTTATG
      1200       1210       1220       1230       1240       1250       1260
AATAATCCACCTGAGATGACTGCACCGCCTGAATCGCCTGAATGATAGCTAATTGTCCACTAGCAGATGC
TTATTAGGTGGACTCTACTGACGTGGCGGACTTAGCGGACTTACTATCGATTAACAGGTGATCGTCTACG
      1270       1280       1290       1300       1310       1320       1330
TCCGCCAACTGTATACGCAAATCCTCTAATATCGAACAGCTCAGTGCACAATCCACAAAGATCGCTATCC
AGGCGGTTGACATATGCGTTTAGGAGATTATAGCTTGTCGAGTCACGTGTTAGGTGTTTCTAGCGATAGG
      1340       1350       1360       1370       1380       1390       1400
ATGAAAGACATTCCATAGTTAGGACGCAAAAAATGAAGCCGATTTTGGTATGCGTTATGCGTTTGGATCA
TACTTTCTGTAAGGTATCAATCCTGCGTTTTTTACTTCGGCTAAAACCATACGCAATACGCAAACCTAGT
      1410       1420       1430       1440       1450       1460       1470
GTTCACGCTGCTGAAAATTAGATCCCCCCACTACTAAACCAATACGATAAGGATCCCATAAAAAGGGCGC
CAAGTGCGACGACTTTTAATCTAGGGGGGTGATGATTTGGTTATGCTATTCCTAGGGTATTTTTCCCGCG
      1480       1490       1500       1510       1520       1530       1540
TGCAAACCTTCCGCCACATTGAAATAAGGGTACACTCCCCCGACTCCGCCATGTATCCAAAATACTGGTC
ACGTTTGGAAGGCGGTGTAACTTTATTCCCATGTGAGGGGGCTGAGGCGGTACATAGGTTTTATGACCAG
      1550       1560       1570       1580       1590       1600       1610
GACCAACAAAGGCATTGTTAAGATGTATAAGTTCTGAGAACTTATTAGACTCTTCAAATATACGCCCTCT
CTGGTTGTTTCCGTAACAATTCTACATATTCAAGACTCTTGAATAATCTGAGAAGTTTATATGCGGGAGA
      1620       1630       1640       1650       1660       1670       1680
ATCTAGAGAGCCCGAAGATTTTTTGATGTTTTCATGATCACTTTTATTTGAATAAAGTTTTTTATCAGAC
TAGATCTCTCGGGCTTCTAAAAAACTACAAAAGTACTAGTGAAAATAAACTTATTTCAAAAAATAGTCTG
      1690       1700       1710       1720       1730       1740       1750
GCCGAAAACTCTTGTGATTGATAAGTATTTTCCCCCAACACCTTGATTTTCATTGATTGGGATATTAATA
CGGCTTTTGAGAACACTAACTATTCATAAAAGGGGGTTGTGGAACTAAAAGTAACTAACCCTATAATTAT
      1760       1770       1780       1790       1800       1810       1820
CGTTGCGGGGTTGTCTCACCATAAAAAAACTTGCAGTCTATTTCCCCACCGCGAACCCCAACCTGAGCTA
GCAACGCCCCAACAGAGTGGTATTTTTTTGAACGTCAGATAAAGGGGTGGCGCTTGGGGTTGGACTCGAT
      1830       1840       1850       1860       1870       1880       1890
TTTCATTTAATTCTTAATCAAAAGCCCTTTTACCCAACCTTTTGTTTGAAAGGTCCCAATCTGATTTCTA
AAAGTAAATTAAGAATTAGTTTTCGGGAAAATGGGTTGGAAAACAAACTTTCCAGGGTTAGACTAAAGAT
      1900       1910       1920       1930       1940       1950       1960
CATTTATTGGGGGGAAAAATATTTGAAATTAAAACCCGCAATGGCCAAAAATTAAAAAGGTTTTTTTTTT
GTAAATAACCCCCCTTTTTATAAACTTTAATTTTGGGCGTTACCGGTTTTTAATTTTTCCAAAAAAAAAA
      1970       1980       1990       2000       2010       2020       2030
GCTTTTAAGAAATTTCTGGGAGAACTTTATTTCTGGAAAATATTAAAAGTTCTATTTAAATTACTTTTTT
CGAAAATTCTTTAAAGACCCTCTTGAAATAAAGACCTTTTATAATTTTCAAGATAAATTTAATGAAAAAA
      2040       2050       2060       2070       2080       2090       2100
TGGGGGGTTTTTTAAAAAAAAGGTGAAAATAAACTGTCCCCACAAATGAAAATATTTTATCTATTTTTGT
ACCCCCCAAAAAATTTTTTTTCCACTTTTATTTGACAGGGGTGTTTACTTTTATAAAATAGATAAAAACA
      2110       2120       2130       2140       2150       2160
GGAAAAAATTTGTTAAGAAAGGCGGGCCATACAACCTTGCCCCCTTAAAACGATTTGTGGGGGTGTTA
CCTTTTTTAAACAATTCTTTCCGCCCGGTATGTTGGAACGGGGGAATTTTGCTAAACACCCCCACAAT
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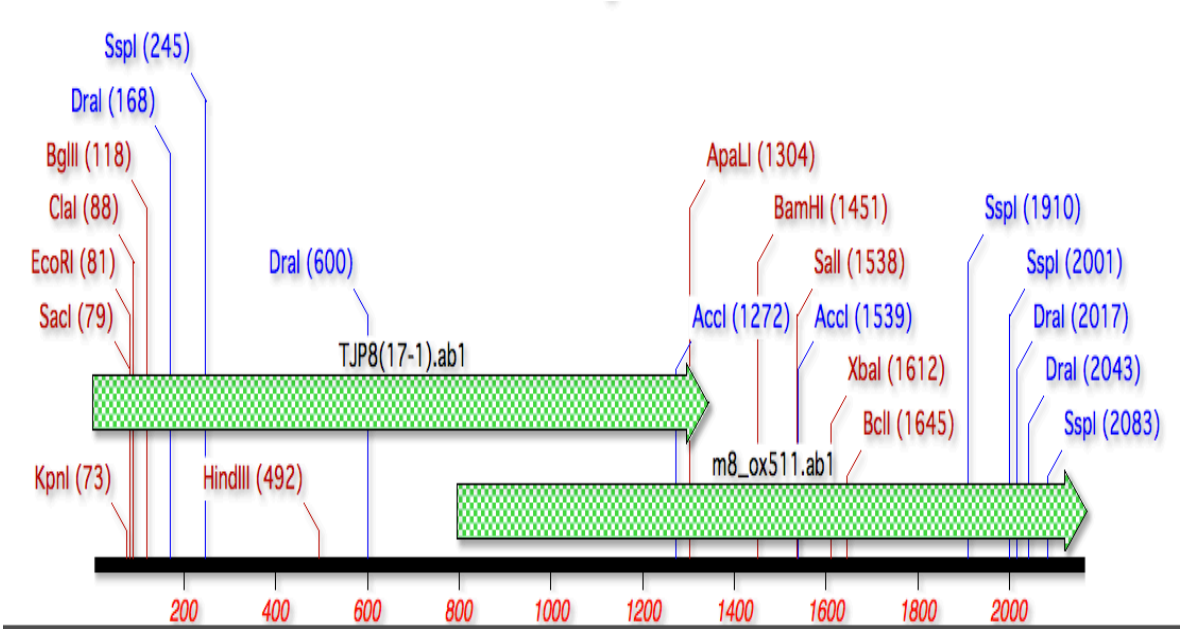

Figure 8.3 Graphic map of sequence contig obtained from Mutant No.8, primer tpnRL17–1 direction. *Bam*H1site identified on the base pair No. 1451. Each green arrow represent the length of sequence coverage and the figure show the level of sequence overlap between each sequence run.

8.2 Appendix (2) Blast results

NCBI Blast:TJP8(17-1) (1241 letters) http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

Description	Max score	Total score	Query cover	E value	Max ident	Accession
3-oxoacyl-(acyl-carrier-protein) synthase II [Serratia odorifera DSM 4582]						
polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica A30] >gi|407752138|gb|EKF62296.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica A30]	68.2	68.2	39%	6e-12	30%	gi|421785523|ZP_16221948.1
polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS12] >gi|333934059|ref|YP_004507637.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica AS9] >gi|386330929|ref|YP_006027099.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS13] >gi|333475666|gb|AEF47376.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica AS9] >gi|333493166|gb|AEF52328.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS12] >gi|333963262|gb|AEG30035.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS13]	68.2	68.2	39%	6e-12	30%	gi|333929106|YP_004502685.1
polyketide synthase [Serratia plymuthica]	64.3	124	39%	1e-10	31%	gi|417353285|AFX60332.1

Figure 8.4 The BLASTX search result obtained from the partial sequencing the flanking genomic DNA from mutant No 8, showing that transposon had inserted into a gene, with similarity to a gene encoding a Polyketide synthase.

NCBI Blast:(2) - TJP7 13-2 (1524 letters) http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Get&AL

Descriptions

Description	Max score	Total score	Query cover	E value	Max ident	Accession
polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS12] >gi|333934059|ref|YP_004507637.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica AS9] >gi|386330929|ref|YP_006027099.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS13] >gi|333475666|gb|AEF47376.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica AS9] >gi|333493166|gb|AEF52328.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS12] >gi|333963262|gb|AEG30035.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia sp. AS13]	34.3	34.3	4%	0.32	57%	gi|333929106|YP_0045026
polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica A30] >gi|407752138|gb|EKF62296.1| polyketide-type polyunsaturated fatty acid synthase PfaA [Serratia plymuthica A30]	34.3	34.3	4%	0.33	57%	gi|421785523|ZP_1622194

Figure 8.5 The blastx search result obtained from the partial sequencing the flanking genomic DNA from mutant No 7(13-2), showing that transposon had inserted into a gene, with similarity to a gene encoding a Polyketide synthase.

NCBI Blast:TJP5(17-1) (1593 letters) http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Get&AI

Range 3: 5226 to 5390

Score	Expect	Method	Identities	Positives	Gaps	Frame
89.0 bits(219)	4e-18()	Compositional matrix adjust.	64/171(37%)	83/171(48%)	15/171(8%)	+1

Features:

```
Query  109   GGFIDDAYCFDPKFFRISPQEAEWMDPQALVLLEESLNVIYHAGYTHHELAGMNVGVYIG  288
             GGFI D   FDP FF ISP+EAE MDPQ  +L+  +  VI  AGY+   LAG  +G+Y+G
Sbjct  5226  GGFIQDMAEFDPLFFGISPREAELMDPQQRLLMTHAWKVIEDAGYSAGALAGSTLGIYVG  5285

Query  289   ARGQQVNLSKIEHCRNP---------IMAVGQNYLAANISQFFNLRGPSLVIDTACSSSL  441
               G       I   + P         + +VG N     +S F N+ GPS  I+TACSSSL
Sbjct  5286  T-GNTGYAGLIVEAKLPAEGFTATGIVPSVGPN-----RMSYFLNVHGPSEPIETACSSSL  5340

Query  442   VGMNMAVGSNVRRYY*LRVGWGG*VCWQIPSAHEIFCRNGNFYYKANRKXF  594
             + ++  V S +R      V  GG      P     FC+ G        K F
Sbjct  5341  IAIHRGV-SALRHEGCDMVIVGGINTLVTPDTFVSFCKAGMLAADGRCKTF  5390
```

Range 4: 645 to 761

Score	Expect	Method	Identities	Positives	Gaps	Frame
82.4 bits(202)	4e-16()	Compositional matrix adjust.	48/121(40%)	66/121(54%)	4/121(3%)	+1

Features:

```
Query  106   RGGFIDDAYCFDPKFFRISPQEAEWMDPQALVLLEESLNVIYHAGYTHHELAGMNVGVYI  285
             RGGF+     FDP FF+ISP+EA+ MDP   +LL+ +   I  AGY   E  G   G++I
Sbjct  645   RGGFMAHIDRFDPLFFKISPREAQLMDPHHRLLLQSAWRAIEDAGYDPQEWHGQQHGIFI  704

Query  286   GARGQQVNLSKIEHCRNPIMAVGQNYLAANISQFFNLRGPSLVIDTACSSSLVGMNMAVG  465
             G   ++ +    EH  + I +V      A I    + +GP L I TACSSSLV ++ A
Sbjct  705   GM--EESDYPLTEH--SAITSVHTGTAPARIGYLLDTKGPLLAIGTACSSSLVALHYACQ  760

Query  466   S  468
             S
Sbjct  761   S  761
```

Figure 8.6 Amino acid sequence line-ups produced from BLASTX between *Serratia plymutica* (P) using the translated mutant 5, sequence contig from primer direction 17-1(Query) and Polyketide synthase, from *Serratia plymuthica* (Sbjct).

NCBI Blast:TJP7 17-1 (1637 letters) http://www.ncbi.nlm.nih.gov/blast/Blast.cg

Descriptions

Description	Max score	Total score	Query cover	E value	Max ident	Accession
beta-ketoacyl synthase [Serratia odorifera 4Rx13] >gi|270043764|gb|EFA16856.1| beta-ketoacyl synthase [Serratia odorifera 4Rx13]	42.7	84.3	11%	0.001	42%	gi|270261888|ZP_06190160.1
polyketide synthase [Serratia marcescens]	42.4	80.9	12%	0.001	42%	gi|417353261|AFX60309.1
polyketide synthase [Serratia plymuthica]	41.6	79.3	14%	0.002	41%	gi|417353285|AFX60332.1
hypothetical protein SOD_b01060 [Serratia odorifera 4Rx13] >gi|270043775|gb|EFA16867.1| hypothetical protein SOD_b01060 [Serratia odorifera 4Rx13]	40.0	113	15%	0.005	36%	gi|270261899|ZP_06190171.1
polyketide synthase [Serratia marcescens]	38.9	73.5	13%	0.013	32%	gi|417353263|AFX60311.1
polyketide synthase [Serratia marcescens]	38.1	73.5	9%	0.023	39%	gi|417353270|AFX60318.1

Figure 8.7 The blastx search result obtained from the partial sequencing the flanking genomic DNA from mutant No 7(17-1), showing that transposon had inserted into a gene, with similarity to a gene encoding a Polyketide synthesis.

Literature Cited in this Thesis

9. Literature cited in this thesis

Ajithkumar, B., (2003). Spore-forming *Serratia marcescens* subsp. sakuensis subsp. nov., isolated from a domestic wastewater treatment tank. International Journal of Systematic and Evolutionary Microbiology **53**: 253–258.

Ahmed, N., Uzair, B., Ayaz, S., and Ahmed, V.U. (2008). Antibacterial activity of marine bacteria from Arabian Sea of Pakistan. The Internet Journal of Microbiology **4**: 1–11.

Alanis, A. J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? Archives of Medical Research **36**: 697-705.

Alström, S., and Gerhardson, B. (1987). Characteristics of a *Serratia plymuthica* isolate from plant rhizospheres. Plant and Soil **103**: 185-189.

Alström, S., and Gerhardson, B. (1988). Differential reactions of wheat and pea genotypes to root inoculation with growth-affecting rhizosphere bacteria. Plant and Soil **109**: 263-269.

Anderson, R.J., Wolf, M.S., and Falkner, D.J. (1974). Autotoxic antibiotic produced by a marine *Chromobacterium*. Marine Biology **27**: 281–285.

Ashelford, K. E., Fry, J. C., Bailey, M. J., and Day, M. J. (2002). Characterization of *Serratia* isolates from soil, ecological implications and transfer of *Serratia proteamaculans* subsp. quinovora Grimont et al. 1983 to *Serratia quinivorans* corrig., sp. nov. International Journal of Systematic and Evolutionary Microbiology **52:** 2281–2289.

Austin, B. (1988). Marine Microbiology. Cambridge: University Press.

Austin, B. (1989). Novel pharmaceutical compounds from marine bacteria. Journal of Applied Microbiology **67**: 461- 470.

Austin, B and Stobie, M. (1992). Recovery of *Serratia plymuthica* and presumptive *Pseudomonas pseudoalcaligenes* from skin lesions in rainbow trout, Oncorhynchus mykiss (Walbaum), otherwise infected with enteric redmouth. Journal of Fish Diseases **15**: 541-543.

Bascomb, S., Lapage, S. P., Willcox, W. R., Curtis, M. A. (1971). Numerical classification of the tribe Klebsielleae. Journal of General Microbiology **66**: 279-295.

Berg, G. (2000). Diversity of antifungal and plant-associated *Serratia plymuthica* strains. Journal of Applied Microbiology **88**: 952-960.

Berg, G., Krechel, A., Ditz, M., Sikora, R. A., Ulrich, A., and Hallmann, J. (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. Federation of European Microbiological Societies Micriobiology Ecology **51**: 215-229.

Berg, G., Kurze, S., Frankowski, J., Richter, I., Dahl, R., Editor: Elad, Y., Kohl, J. and Shtienberg, D. (2002). Biocontrol agent *Serratia plymuthica* strain HRO-C48: performance in relation to environmental factors. In: Duffy BK, Rosenberger U, Défago G (eds) Molecular Approaches in Biological Control, IOBC/wprs, Bulletin **25**:111-114.

Bernan, V., Greenstein, M., and Maiese, W. (1997). Marine Microorganisms as a Source of New Natural Products. Advances in Applied Microbiology **43**: 57-90.

Bhadra, B., Roy, P., and Chakraborty, R. (2005). *Serratia ureilytica* sp. nov., a novel urea-utilizing species. International Journal of Systematic and Evolutionary Microbiology **55**: 2155–2158.

Bizio, B. (1823). Lettera di Bartolomeo Bizio al chiarissimo canonico Angelo Bellani sopra il fenomeno della polenta porporina. Biblioteca Italiana o sia Giornale di Letteratura Scienze a Arti **30**:275–295.

Breed, R. S., Murray, E. G. D., Hitchens, A. P. (1948). Bergey's Manual of Determinative bacteriology. 6th ed. Baltimore, Williams and Wilkins.

Bryskier, A. (2005). Antimicrobial agents: antibacterials and antifungals. The American Society for Microbiology Press, Washington, USA.

Burgess, J. G., Jordan, E. M., Bregu, M., Mearns-Spragg, A., and Boyd, K. G. (1999). Microbial antagonism: a neglected avenue of natural products research. Journal of Biotechnology **70**: 27-32.

Burkholder, P. R., Pfister, R. M., and Leitz, F. H. (1966). Production of a pyrrole antibiotic by a marine bacterium. Applied Microbiology **14**: 649-653.

Calvente, V., De Orellano, M. E., Sansone, G., Benuzzi, D., and Sanz de Tosetti, M. I. (2001). Effect of nitrogen source and pH on siderophore production by *Rhodotorula* strains and their application to biocontrol of phytopathogenic moulds. Journal of Industrial Microbiology and Biotechnology **26**: 226-229.

Carlot, M., Giacomini, A., and Casella, S. (2002). Aspects of Plant-Microbe Interactions in Heavy Metal Polluted Soil. Acta Biotechnologica **22**: 13-20.

Carrero, P., Garrote, J. A., Pacheco, S., García, A. I., Gil, R., and Carbajosa, S. G. (1995). Report of six cases of human infection by *Serratia plymuthica*. Journal of Clinical Microbiology **33**: 275-276.

Charrier, M., Fonty, G., Gaillard-Martinie, B., Ainouche, K., and Andant, G. (2006). Isolation and characterization of cultivable fermentative bacteria from the intestine of two edible snails, *Helixpomatia* and Cornu *aspersum* (*Gastropoda*: *Pulmonata*). Biological Research **39**: 669-681.

Chernin, L, Ismailov, Z., Haran, S., and Chet, I. (1995). Chitinolytic *Enterobacter agglomerans* Antagonistic to Fungal Plant Pathogens. Applied and Environmental Microbiology **61**: 1720-1726.

Chernin, Leonid, Brandis, A., Ismailov, Z., and Chet, I. (1996). Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. Current microbiology **32**: 208-212.

Compant, S., Duffy, B., Nowak, J., and Cle, C. (2005). Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases : Principles , Mechanisms of Action , and Future Prospects. Applied and Enviromental Microbiology **71**: 4951- 4959.

Davidson, B. S. (1995). New dimensions in natural products research: cultured marine microorganisms. Current Opinion in Microbiology **6**: 284-291.

Davies, J., and Davies, D. (2010). Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews **74**: 417- 433.

DeLisle, S., and Perl, T. (2003). Vancomycin-resistant *enterococci*: a road map on how to prevent the emergence and transmission of antimicrobial resistance. CHEST Journal **123**: 5045-5185.

Dessen, A., Di, Guilmi A., Vernet, T., and Dideberg, O. (2001). Molecular Mechanisms of Antibiotic Resistance in Gram-Positive Pathogens. Current Drug Targets Infectious Disorders **1**: 63-77.

Dharmaraj, S. (2010). Marine *Streptomyces* as a novel source of bioactive substances. World Journal of Microbiology and Biotechnology **26**: 2123-2139.

Espinal, M., and Laszlo, A. (2001). Global trends in resistance to antituberculosis drugs. The New England Journal of Medicine **344**: 1294-1303.

Ewing, W. H., Davis, B. R., Fife, M. A., and Lessel, E. F. (1973). Biochemical characterization of *Serratia liquefaciens* (Grimes and Hennerty) Bascomb et al. (formerly *Enterobacter liquefaciens*) and *Serratia rubidea* (Stapp) comb. nov. and designation of type and neotype strains. International Journal of Systematic Bacteriology **23**: 217-225.

Faltin, F., Lottmann, J., Grosch, R., and Berg, G. (2004). Strategy to select and assess antagonistic bacteria for biological control of *Rhizoctonia solani* Kühn. Canadian Journal of Microbiology **50**: 811-820.

Farmer, J. (1985). Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. Journal of Clinical Microbiology **21**: 46-76.

Fey, P., and Safranek, T. (2000). Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. The New England Journal of Medicine **342**: 1242-1249.

Frankowski, J., Berg, G., Bahl, H., Duffy, B., Rosenberger, U., and Defago, G. (1998). Mechanisms involved in the antifungal activity of the rhizobacterium *Serratia plymuthica*. In: Duffy BK, Rosenberger U, Défago G (eds) Molecular Approaches in Biological Control, IOBC/wprs, Bulletin **21**: 45-50.

Frankowski, J., Lorito, M., Scala, F., Schmid, R., Berg, G., and Bahl, H. (2001). Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. Archives of Microbiology **176**: 421-426.

Frommel, M.I., Pazos, G.S., and Nowak, J. (1991). Plant-growth stimulation and biocontrol of Fusarium wilt (*Fusarium oxysporum f.* sp. *lycopersici*) by co-inoculation of tomato seeds with *Serratia plymuthica* and *Pseudomonas* sp. Journal Fitopatologia Brasileira **26**: 66-73.

Galland, D., and Paul, B. (2001). *Pythium perplexum* isolated from soil in France: morphology, molecular characterisation and biological control. Microbiological Research **156**: 185-189.

Gauthier, M.J. and Flateau, G.N. (1976). Antibacterial activity of marine violet-pigmented *Alteromonas* with special reference to the production of brominated compounds. Canadian Journal of Microbiology **22**: 1612-1619

Gavini, F., Ferragut, C., Izard, D., Trinel, P. A., Leclerc, H., Lefebvre, B., and Mossel, D. A. A. (1979). *Serratia fonticola*, a new species from water. International Journal of Systematic Bacteriology **29**: 92–101.

Geiger, A., Fardeau, M.L., Falsen, E., Olliver, B., and Cuny, G. (2010). *Serratia glossinae* sp. nov., Isolated from the midgut of the tsetse fly Glossina palpalis gambiensis. International Journal of Systematic and Evolutionary Microbiology **60**: 1261-1265.

Gerard, J. M., Haden, P., Kelly, M. T., and Andersen, R. J. (1999). Loloatins A-D, cyclic decapeptide antibiotics produced in culture by a tropical marine bacterium. Journal of Natural Products **62**: 80-85.

Grimes, M., and Hennerty, A. J. (1931). A study of bacteria belonging to the sub-genus *Aerobacter.* Scientific Proceedings of the Royal Dublin Society, New Series. **20**: 89-97.

Grimont, P., and Grimont, F. (1978). The genus *Serratia*. Annual Review of Microbiology **32**: 221- 248.

Grimont, P. A. D., Grimont, F. (1981). The genus *Serratia* (1187–1203). In: Starr, M. P., Stolp, H., Truper, H. G., Balows, A., and Schlegel, H. G. (ed.), The prokaryotes: A handbook on habitats, isolation, and identification of bacteria. Springer-Verlag. Berlin.

Grimont, P. A. D., Grimont, F., and Irino, K. (1982). Biochemical characterization of *Serratia liquefaciens* sensu stricto*, Serratia proteamaculans,* and *Serratia grimesii* sp. nov. Current Microbiology **7**: 69-74.

Grimont, P. A. D., Grimont. F., Richard, C., Davis. B.R., Steigerwalt. A. G and Brenner, D. J. (1978). Deoxyribonucleic acid relatedness between *Serratia plymuthica* and other *Serratia* species, with a description of *Serratia odorifera* sp. nov.(type strain: ICPB 3995). International Journal of Systematic and Evolutionary Microbiology **28**, 453-463.

Grimont, P. A. D., Grimont, F., and Starr, M. P. (1981). *Serratia* species isolated from plants. Current Microbiology **5**: 317-322.

Grimont, P. A. D., Jackson, T. A., Ageron, E., and Noonan, M. J. (1988). *Serratia entomophila* sp. nov. associated with amber disease in the New Zealand grass grub Costelytra zealandica. International Journal of Systematic Bacteriology **38**:1–6.

Harris, A. K., Williamson, N. R., Slater, H., Cox, A., Abbasi, S., Foulds, I., Simonsen, H. T., Leeper, F. J., and Salmond, G. P. (2004). The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows species- and strain-dependent genome context variation. Microbiology **150**: 3547-3560.

Herbert, R. B. (1981). The Biosynthesis of Secondary Metabolites (ist edn.). USA: Chapman and Hall.

Hofte, M., Buysens, S., Koedam, N., and Cornelis, P. (1993). Zinc affects siderophore-mediated high affinity iron uptake systems in the rhizosphere *Pseudomonas aeruginosa* 7NSK2. Biometals an International Journal on the Role of Metal Ions in Biology Biochemistry and Medicine **6**: 85-91.

Hucker, G. J., and Conn, H. J. (1923). Methods of Gram straining. In Technical Bulletin of the New York State of Agricultural and Experimental Station No. 93

Imamura, N., Nishijima, M., Takadera, T., Adachi, K., Sakai, M., and Sano, H. (1997). New anticancer antibiotics pelagiomicins, produced by a new marine bacterium *Pelagiobacter variabilis*. The Journal of Antibiotics (Tokyo) **50**: 8-12.

Jaffe, H., Biddle, J., Johnson, S., and Wiesner, P. (1981). Infections Due to Penicillinase-Producing *Neisseria gonorrhoeae* in the United States: 1976-1980. The Journal of Infectious Diseases **144**: 19761-1980.

Jorgensen, J. H. (1992). Update on mechanisms and prevalence of antimicrobial resistance in *Haemophilus influenzae*. Clinical Infectious Disease*s* **14**: 1119-1123.

Kaiser, D., Onken, U., Sattler, I., and Zeeck, A. (1994). Influence of increased dissolved oxygen concentration on the formation of secondary metabolites by manumycin-producing *Streptomyces parvulus*. Applied Microbiology and Biotechnology **41**: 309-312.

Kalbe, C., Marten, P., and Berg, G. (1996). Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. Microbiological Research **151**: 433-439.

Kamensky, M., Ovadis, M., Chet, I., and Chernin, L. (2003). Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. Soil Biology and Biochemistry **35**: 323-331.

Khosla, C., Gokhale, R. S., Jacobsen, J. R., and Cane, D. E. (1999). Tolerance and specificity of polyketide synthases. Annual Review of Biochemistry **68**: 219-253.

Kirner, S., Hammer, P. E., Hill, D. S., Altmann, a, Fischer, I., Weislo, L. J., Lanahan, M., and *et al*. (1998). Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. Journal of Bacteriology **180**:1939–1943.

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. Biochimie **70** : 337–349.

Kraemer, S. M. (2004). Iron oxide dissolution and solubility in the presence of siderophores. Aquatic Sciences Research Across Boundarie*s* **66**: 3–18.

Krausse, R., Piening, K., and Ullmann, U. (2005). Inhibitory effects of various micro-organisms on the growth of *Helicobacter pylori*. Letters in Applied Microbiology **40**: 81–86.

Kunze, B., Jansen, R., Sasse, F., Hofle, G., and Reichenbach, H. (1995). Chondramides A approximately D, new antifungal and cytostatic depsipeptides from *Chondromyces crocatus* (myxobacteria). Production, physico-chemical and biological properties. The Journal of Antibiotics (Tokyo) **48**: 1262-1266.

Kurze, S., Bahl, H., and Dahl, R. (2001). Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. Plant Disease **85**: 529-534.

Labbate, M., Queck, S., and Koh, K. (2004). Quorum Sensing-Controlled Biofilm Development in *Serratia liquefaciens* MG1. Journal of Bacteriology **186**: 692-698.

Lane, D. J. (1991). 16S/23S rRNA sequencing. Wiley.

Larsen, R. a, Wilson, M. M., Guss, A. M., and Metcalf, W. W. (2002). Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Archives of Microbiology **178**: 193-201.

Lehmann, K. B., Neumann, R. (1896). Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik, Teil II. Munich, Lehmann.

Leifert, C., Sigee, D. C., Stanley, R., Knight, C., and Epton, H. A. S. (1993). Biocontrol of Botrytis cinerea and Alternaria brassicicola on Dutch white cabbage by bacterial antagonists at cold-store temperatures. Plant Pathology **42**: 270-279.

Lemos, M.l., Toranzo, A.E., and Barja, J. L. (1985) Antibiotic activity of epiphytic bacteria isolated fr om intertidal seaweeds. Microbial Ecology **11**: 149-163.

Levenfors, J. J., Hedman, R., Thaning, C., Gerhardson, B., and Welch, C. J. (2004). Broad-spectrum antifungal metabolites produced by the soil bacterium *Serratia plymuthica* A 153. Soil Biology and Biochemistry **36**: 677- 685.

Liu, X., Bimerew, M., Ma, Y., Müller, H., Ovadis, M., Eberl, L., Berg, G., andc *et al*., (2007). Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*. Federation of European Microbiological Societies Microbiology Letters **270**: 299–305.

Loper, J. E., and Henkels, M. D. (1999). Utilization of Heterologous Siderophores Enhances Levels of Iron Available to *Pseudomonas putida* in the Rhizosphere. Applied and Environmental Microbiology **65**: 5357-5363.

Lovell, F. M. (1966). The structure of a bromine-rich antibiotic. Journal of the American Chemical Society **88**: 510-4511.

Lowy FD. (1998). Medical progress: *Staphylococcus aureus* infections. The New England Journal of Medicine **339**: 520-532.

Lucon, C. M. M. and Melo, I. S. (2000). Effect of seed bacterization on the development of maize plants and *Fusarium moniliforme* control. Journal Fitopatologia Brasileira **25**: 529-537.

Martin, J. F., and Demain, A. L. (1980). Control of Antibiotic Biosynthesis. Microbiological Reviews **44**: 230-251.

Martín, J., and Gil, J. (1984). Cloning and expression of antibiotic production genes. Nature Biotechnology **2**: 63-72

Marwick, J., Wright, P., and Burgess, J. (1999). Bioprocess Intensification for Production of Novel Marine Bacterial Antibiotics Through Bioreactor Operation and Design. Marine Biotechnology **1**: 495-507.

Matilla, M.A., Stöckmann, H., Leeper, F.J., and Salmond, G.P.C. (2012). Bacterial biosynthetic gene clusters encoding the anti-cancer haterumalide class of molecules: biogenesis of the broad spectrum antifungal and anti-oomycete compound, oocydin A. The Journal of Biological Chemistry **287**: 39125-39138.

McCullagh, M., Utkhede, R., Menzies, J. G., Punja, Z. K., and Paulitz, T. C. (1996). Evaluation of plant growth-promoting rhizobacteria for biological control of Pythium root rot of cucumbers grown in rockwool and effects on yield. European Journal of Plant Pathology **102**: 747-755.

Mearns-Spragg, a, Bregu, M., Boyd, K. G., and Burgess, J. G. (1998). Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. Letters in Applied Microbiology **27**: 142-146.

Meziane, H., Gavriel, S., Ismailov, Z., Chet, I., Chernin, L., and Hofte, M. (2006). Control of green and blue mould on orange fruit by *Serratia plymuthica* strains IC14 and IC1270 and putative modes of action. Postharvest Biology and Technology **39**: 125-133.

Miethke, M., and Marahiel, M. A. (2007). Siderophore-based iron acquisition and pathogen control. Microbiology and Molecular Biology Reviews **71**: 413- 451.

Miller, M. B., and Bassier B. L. (2001). Quorum sensing in bacteria. Annual Reviews in Microbiology **55**: 165-199.

Minowa, Y., Araki, M., and Kanehisa, M. (2007). Comprehensive analysis of distinctive polyketide and nonribosomal peptide structural motifs encoded in microbial genomes. Journal of Molecular Biology **368**: 1500-1517.

Moons, P., Van Houdt, R., Aertsen, A., Vanoirbeek, K., Engelborghs, Y., and Michiels, C. W. (2006). Role of quorum sensing and antimicrobial component production by *Serratia plymuthica* in formation of biofilms, including mixed biofilms with *Escherichia coli*. Applied and Environmental Microbiology **72**: 7294-7300.

Neupane, S., Finlay, R. D., Kyrpides, N. C., Goodwin, L., Alström, S., Lucas, S., Land, M., Han, J., Lapidus, A., Cheng, J.F., Bruce, D., Pitluck, S., Peters, L., Ovchinnikova, G., Held, B., Han, C., Detter, J.C., Tapia, R., Hauser, L., Ivanova, N., Pagani, I., Woyke, T., Klenk, H.P., and Högberg N La. (2012). Complete genome sequence of the plant-associated *Serratia plymuthica* strain AS13. Standards in Genomic Sciences **7**: 22-30.

Nieto, T. P., Rodríguez L. A., Santos, Y., Núñez, S., and Toranzo, A. E. (1990). Isolation of *Serratia plymuthica* as an opportunistic pathogen in rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Diseases**13**: 175-177.

Nosova, T., Jokelainen, K., Kaihovaara, P., Jousimies-Somer, H., Siitonen, A., Heine, R., and Salaspuro, M. (1996). Aldehyde dehydrogenase activity and acetate production by aerobic bacteria representing the normal flora of human large intestine. Alcohol and Alcoholism Oxford Oxfordshire **31**; 555–564.

Nowak-Thompson, B., Chaney, N., Wing, J. S., Gould, S. J., and Loper, J. E. (1999). Characterization of the Pyoluteorin Biosynthetic Gene Cluster of *Pseudomonas fluorescens* Pf-5. Journal of Bacteriology **181**: 2166-2174.

Nuorti, J. P., Butler, J. C., Crutcher, J. M., Guevara, R., Welch, D., Holder., and Elliott J. A. (1998). An Outbreak of Multidrug Resistant *Pneumococcal Pneumonia* and Bacteremia among Unvaccinated Nursing Home Residents. The New England Journal of Medicine **338**:1861-1868.

O'Sullivan, D. J., and O'Gara, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiological Reviews **56**: 662-676.

Okami, Y. (1986). Marine microorganisms as a source of bioactive agents. Microbial Ecology **12**: 65-78.

Ovadis, M., Liu, X., Gavriel, S., Ismailov, Z., Chet, L., and Chernin, L. (2004). The global regulator genes from biocontrol strain *Serratia plymuthica* IC1270: cloning, sequencing, and functional studies. Journal of Bacteriology **186**: 4986- 4993.

Paine, S. G., Berridge, E. M. (1921). Studies in bacteriosis. V. Further investigation of a suggested bacteriolytic action in Protea cynaroides affected with the leaf-spot disease. Annals of Applied Biology **8**: 20–26.

Pang, Y., Liu, X., Ma, Y., Chernin, L., Berg, G., and Gao, K. (2008). Induction of systemic resistance, root colonisation and biocontrol activities of the rhizospheric strain of *Serratia plymuthica* are dependent on N-acyl homoserine lactones. European Journal of Plant Pathology **124**: 261-268.

Park.C and Shen.S. (2002). Biocontrol of phytophthora blight of pepper employing *Serratia plymuthica* A21–4 and effect of soil population of Phytophthora capsici on the root colonization of the antagonistic bacteria. In: Duffy BK, Rosenberger U, Défago G (eds) Molecular Approaches in Biological Control, IOBC/wprs, Bulletin **25**: 327-30.

Patil, R., Ghormade, V., and Deshpande, M. (2000). Chitinolytic enzymes: an exploration. Enzyme and Microbial Technology **26**: 473-483.

Patrick A. D. Grimont,t Francine Grimont, t and M. P. S. (1981). *Serratia* Species Isolated from Plants. Current Microbiology **5**: 317-322.

Pfeifer, B. A., Wang, C. C. C., Walsh, C. T., and Khosla, C. (2003). Biosynthesis of Yersiniabactin, a complex polyketide-nonribosomal peptide, using *Escherichia coli* as a heterologous host. Applied and Environmental Microbiology **69**: 6698-6702.

Pusecker, K., Laatsch, H., Helmke, E., and Weyland, H. (1997). Dihydrophencomycin methyl ester, a new phenazine derivative from a marine *Streptomycete*. The Journal of Antibiotics **50**: 479-483.

Rangaswamy, V., Jiralerspong, S., Parry, R., and Bender, C. L. (1998). Biosynthesis of the *Pseudomonas* polyketide coronafacic acid requires monofunctional and multifunctional polyketide synthase proteins. Proceedings of the National Academy of Sciences of the United States of America **95**: 15469-15474.

Ritte, E., Lurie, S., Droby, S., Ismailov, Z., Chet, I., and Chernin, L. (2002). Biocontrol of postharvest fungal pathogens of peaches and apples by *Pantoae agglomerans* strain IC1270. In: Duffy BK, Rosenberger U, Défago G (eds) Molecular Approaches in Biological Control, IOBC/wprs, Bulletin **25**: 199-202.

Sabri, A., Leroy, P., Haubruge, E., Hance, T., Frère, I., Destain, J., and Thonart, P.(2011). Isolation, pure culture and characterization of *Serratia symbiotica* sp. nov., the R-type of secondary endosymbiont of the black bean aphid Aphis fabae. International Journal of Systematic and Evolutionary Microbiology **61:** 2081–2088.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory.

Schwarzer, D., Finking, R., and Marahiel, M. A. (2003). Nonribosomal peptides: from genes to products. Natural Product Reports **20**: 275-287.

Seppälä, H., Klaukka, T., Lehtonen, R., Nenonen, E., and Huovinen, P. (1995). Outpatient use of erythromycin: link to increased erythromycin resistance in group A *Streptococci*. Clinical Infectious Diseases **21**: 1378-1385.

Shen, S., Piao, F., Lee, B., and Park, C. (2007). Characterization of Antibiotic Substance Produced by *Serratia plymuthica* A21-4 and the Biological Control Activity against Pepper Phytophthora Blight. The Plant Pathology Journal **23**: 180-186.

Shiozawa, H., Kagasaki, T., Kinoshita, T., Haruyama, H., Domon, H., Utsui, Y., Kodama, K., and Takahashi. S. (1993). Thiomarinol, a new hybrid antimicrobial antibiotic produced by marine bacterium. The Journal of Antibiotics **46**: 1834-1841.

Shoji, J., Hinoo, H., Sakazaki, R., Kato, T., Hattori, T., Matsumoto, K., Tawara, K., Kikuchi, J., and Terui, Y. (1989). Isolation of CB-25-I, an antifungal antibiotic, from *Serratia plymuthica*. The Journal of Antibiotics **42**: 869-874.

Skerman, V. B. D., McGowan, V., Sneath, P. H. A. (1980). Approved lists of bacteria names. International Journal of Systematic Bacteriology **30**: 225- 420.

Sleigh, J. D. (1983). Antibiotic resistance in *Serratia marcescens*. Brithish Medical Journal **287**: 1651–1653.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology **98**: 503–517.

Spížek, J., and Tichý, P. (1995). Some aspects of overproduction of secondary metabolites. Folia microbiologica **40**: 43-50.

Stach, J., (2010). Antimicrobials: treasures from the oceans. Microbiology Today **37**: 104 -108.

Stapp, C., (1940). *Bacterium rubidaeum* nov. spec. Zentralbl. Journal Bakteriol **102**: 251-260.

Stephen J. Wratten, Margaret S. Wolfe, Raymond J. Andersen, and D. J. F. (1977). Antibiotic metabolites from a marine pseudomonad. Antimicrobial agents and Chemotherapy **11**: 411- 414.

Stock, I., Burak, S., Sherwood, K. J., Gruger, T.,and Wiedemann, B. (2003). Natural antimicrobial susceptibilities of strains of "unusual" *Serratia* species: *S. ficaria, S. fonticola, S. odorifera, S. plymuthica* and *S. rubidaea*. The Journal of Antimicrobial chemotherapy **51**: 865-885.

Swift, S., Karlyshev, A. V, Fish, L., Durant, E. L., Winson, M. K., Chhabra, S. R. A. M., Williams, P. (1997). Quorum Sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: Identification of the LuxRI Homologs AhyRI and AsaRI and Their Cognate N-Acylhomoserine Lactone Signal Molecules. Journal of Bacteriology **179**: 5271–5281.

Thaning, C., Welch, C. J., Borowicz, J. J., Hedman, R., and Gerhardson, B. (2001). Suppression of Sclerotinia sclerotiorum apothecial formation by the soil bacterium *Serratia plymuthica* : identification of a chlorinated macrolide as one of the causal agents. Soil Biology and Biochemistry **33**: 1817-1826.

Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A., and Salmond, G. P. (2000). Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia i*s under quorum sensing control. Molecular Microbiology **36**: 539–556.

Tiwari, H. K., and Sen, M. R. (2006). Emergence of vancomycin resistant *Staphylococcus aureus* (VRSA) from a tertiary care hospital from northern part of India. BMC Infectious Diseases **6**: 1-6.

Van Houdt, R., Givskov, M., and Michiels, C. W. (2007a). Quorum sensing in *Serratia*. Federation of European Microbiological Societies Microbiology Reviews **31**: 407-24.

Van Houdt, R., Moons, P., Aertsen, A., Jansen, A., Vanoirbeek, K., Daykin, M., Williams, P., and Michiels, C. (2007b). Characterization of a luxI/luxR-type quorum sensing system and N-acyl-homoserine lactone-dependent regulation of exo-enzyme and antibacterial component production in *Serratia plymuthica* RVH1. Research in Microbiology **158**: 150-158.

Van Houdt, R., Moons, P., Jansen, A., Vanoirbeek, K., and Michiels, C. W. (2006). Isolation and functional analysis of luxS in *Serratia plymuthica* RVH1. Federation of European Microbiological Societies Microbiology Letters **262**: 201-209.

Vivas, J., González, J. a, Barbeyto, L., and Rodríguez, L. A. (2000). Identification of environmental *Serratia plymuthica* strains with the new combo panels type 1S. Memórias do Instituto Oswaldo Cruz **95**: 227-229.

Vleesschauwer, D., and Hofte, M. (2007). Using *Serratia plymuthica* to control fungal pathogens of plants. CAB Reviews **2**:1-12.

Vucemilo, M., Vinkovic, B., Tofant, A., Simpraga, B., Pavicic, Z., and Matkovic, K. (2005). Microbiological air contamination in intensive poultry breeding. International Society for Animal Hygiene (Warsaw, Poland) **2**: 127-129.

Waterer, G.W., and Wunderink, R. G. (2001). Increasing threat of Gram-negative bacteria. Critical Care Medicine **29**:75-81.

Waters, C. M., and Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. Annual Review of Cell and Developmental Biology **21**: 319-346.

Wratten, S.J., Wolfe, M.S., Anderson, R.J., and Faulkner, D.J. (1977). Antibiotic metabolites from a marine pseudomonad. Antimicrobial Agents and Chemotherapy **11**: 411- 414.

Wright, A. E and McCarthy, P. J. (1994). Drugs from the sea at Harbor Branch. Sea Technology **35**: 10-19.

Wright, S. A. I., Zumoff, C. H., Schneider, L., and Beer, S. V. (2001). *Pantoea agglomerans* Strain EH318 Produces Two Antibiotics That Inhibit *Erwinia amylovora* In Vitro. Applied and Environmental Microbiology **67** (284–292).

Xue, S., Zhang, H.T., Wu, P.C., Zhang, W., and Yuan, Q. (2003). Study on biodiversity of extracts from marine sponges in Chinese Sea. Journal of Experimental Marine Biology and Ecology **298**: 71-78.

Yan, L., Boyd, K. G., Adams, D. R., and Burgess, J. G. (2003). Biofilm-specific cross-species induction of antimicrobial compounds in bacilli. Applied and Environmental Microbiology **69**: 3719-3727.

Zhang, C.-X., Yang, S.-Y., Xu, M.-X., Sun, J., Liu, H., Liu, J.-R., Liu, H. (2009). *Serratia nematodiphila* sp. nov., associated symbiotically with the entomopathogenic nematode *Heterorhabditidoides chongmingensis* (*Rhabditida*: *Rhabditidae*). International Journal of Systematic and Evolutionary Microbiology **59**:1603–1608.

Zbinden, R., and Blass, R. (1988). *Serratia plymuthica* osteomyelitis following a motorcycle accident. Journal of Clinical Microbiology **26**: 1409-1410.

Zheng, L., Han, X., Chen, Lin, H., Lin, W., and Yan, X. (2005). Marine bacteria associated with marine macroorganisms: the potential antimicrobial resources. Annals of Microbiology **55**: 119-124.

ZoBell, C. E., and Upham, H. C. (1944). A list of marine bacteria including descriptions of sixty new species. Bulletin of the Scripps Institution of Oceanography **5**: 239-281.

Web pages:

1. BLAST: Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/).

2. Centers for Disease Control and Prevention (http://www.cdc.gov).

3. Formerly List of Bacterial names with Standing in Nomenclature (LBSN) (http://www.bacterio.cict.fr/s/serratia.html).

4. GE Healthcare Life Sciences (http://www.gelifesciences.com/).

5. Thermo scientific (http://www.thermoscientificbio.com/fermentas/).

6. The world Economic Forum (http://forumblog.org/tag/antibiotics).